Original Article

Correlation of Primary Hepatocellular Carcinoma with HBV Genotypes, Subgenotypes and Gene Mutations in Gansu Province

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Objective To investigate the occurrence of basal core promoter (BCP) and pre-C mutations in patients with hepatitis B virus (HBV) infection in Gansu Province, China, and to analyze the correlation of HBV mutation and HBV genotype with primary hepatocellular carcinoma (HCC).

Methods PCR-RFLP was applied to detect HBV subgenotypes, and the presence of the pre-C and BCP mutations in 62 patients with HCC, 70 patients with hepatitis B induced liver cirrhosis (LC) and 90 patients with chronic hepatitis B (CHB).

Results In HCC patients, genotype C was the major genotype (70.97%). The pre-C mutation was found in 59.68%, 31.43% and 16.67% patients with HCC, LC and CHB, respectively. The frequency of BCP mutations was significantly different between patients with HCC, LC and CHB (74.19%, 51.43% and 37.78%, respectively; $\chi^2=30.727$, 19.540, respectively, $P < 0.01$). Patients in HCC group had a higher incidence of pre-C as well as BCP mutations compared to the other groups. The prevalence of pre-C and BCP mutations was significantly higher in patients with genotype C1 (44.32% and 69.32%, respectively) compared to patients with other subgenotypes ($P < 0.05$).

Conclusions The incidence of pre-C and BCP mutations increases with disease progression. Pre-C and BCP mutations frequently occur in patients with genotype C1. HBV genotype C, pre-C mutations and BCP mutations are closely related to the occurrence of HCC.

Key words: Hepatitis B virus; Hepatocellular carcinoma; Genotype; Subgenotype; Mutation

Hepatocellular carcinoma (HCC), a common malignancy in China,¹ has a high prevalence and poor prognosis and is a significant threat to human health. Infection with the hepatitis B virus (HBV) is major risk factor for the occurrence and development of HCC. The major genotypes of HBV in China are genotypes B and C. Genotype B is mainly found in South China and genotype C in North China. A number of studies have investigated the role of HBV mutation: 1) the A to T substitution at nucleotide 1762 of the hepatitis B virus basic core promoter (HBV BCP) region; 2) the G to A substitution at nucleotide 1764 of the HBV BCP

region; 3) the pre-C stop codon mutation (A1896) of HBV, acting on the biological characteristics of HBV. The incidence of the BCP 1762/1764 mutation was recently reported to occur frequently in HCC patients, although this is still controversial. This study was undertaken to detect the BCP mutation and pre-C mutation in 222 patients with chronic HBV infection, and to analyze the correlation of HBV mutation, HBV genotypes and subgenotypes with HCC.

PATIENTS AND METHODS

Patients

Blood was collected from 222 patients diagnosed with chronic HBV infection at the Department of Infectious Diseases of the Second People's Hospital in Gansu Province, China, from September, 2005 to September, 2011. Serum was obtained after

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centrifugation. The study population consisted of 186 males and 36 females, with a mean age of 35.5 ± 11.6 years (range: 6-76 years). There were 90 patients with chronic hepatitis B (CHB), 70 patients with post-hepatic cirrhosis and 62 patients with HCC. Patients with HCV infection and concomitant infection of other hepatitis related viruses were excluded. Patients with other liver diseases including alcoholic liver disease, hereditary metabolic liver disease, autoimmune hepatitis and drug-induced liver injury were also excluded. The diagnosis of chronic hepatitis and liver cirrhosis was made according to the criteria in "the Guideline for Prevention and Treatment of Viral Hepatitis in 2010".¹ The diagnosis of HCC was done according to the diagnostic criteria developed in the American Liver Diseases Annual Meeting in 2005.²

Methods

Extraction of genomic DNA

Venous blood (2 ml) was collected in the presence of EDTA. Total genomic DNA was extracted using the UNIQ210 column whole blood genomic extraction kit (Shanghai Sangon Biotech Co., Ltd) according to the manufacturer's instructions.

Design and synthesis of primers

Primers were designed as previously reported and synthesized by Shanghai Sangon Biotech Co., Ltd.

Genotyping of HBV

PCR-RFLP was performed as previously reported.³ In brief, PCR was performed using 2 µl HBV DNA and primers. Reaction conditions were as follows: pre-denaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 25 s, and extension at 72°C for 50 s and a final extension at 72°C for 5 min. PCR products (2 µl) were then subjected to nested PCR in a 30 µl mixture. The reaction conditions were as follows: predenaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 25 s and extension at 72°C for 40 s, and a final extension at 72°C for 5 min. The PCR products were subjected to digestion with *Bsrs* I and *Sty* I (3 U) restriction endonucleases, and 10 µl of the digestion products were subjected to electrophoresis on 2.5% agarose gels. The representative images were input into a computer and the spectrum was used for genotyping. PCR products not digested by *Bsrs* I and *Sty* I restriction endonucleases were digested with *Alw* I restriction endonuclease. When all three restriction endonucleases failed to identify

the genotype, the PCR products were subjected to sequencing in order to determine the genotype.

Analysis of subgenotypes

Determination of subgenotype C

Subgenotype C determination was performed as described previously.⁴ HBV DNA (2 µl) was mixed with primers in a total reaction volume of 20 µl. The reaction conditions were as follows: predenaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 20 s and extension at 72°C for 40 s, and a final extension at 72°C for 5 min. PCR products (2 µl) were subjected to semi-nested PCR in a total reaction volume of 30 µl. The reaction conditions for the semi-nested PCR were as follows: predenaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 25 s and extension at 72°C for 35 s, and a final extension at 72°C for 5 min. The PCR products were digested with 3 U each of *Ase* I and *Nci* I endonucleases. Digestion products (10 µl) were subjected to electrophoresis on 2.5% agarose gels. A gel image analysis system was used to capture the images and the spectrum was used for genotyping. PCR products not digested by *Ase* I and *Nci* I were subjected to digestion with *BesE* II endonuclease. Amplification of pre-C/C region of HBV was carried out when the genotype was not confirmed, followed by sequencing to determine the subgenotype.

Determination of subgenotype B

Subgenotype B determination was performed as described previously.⁵ PCR products were subjected to digestion with *Stu* I endonuclease (3 U). Digestion products (10 µl) were subjected to electrophoresis on 2.5% agarose gels. A gel image analysis system was applied to capture the images and the spectrum was used for genotyping. PCR products not digested by *Stu* I restriction endonuclease were subjected to digestion with *Hpa* I restriction endonuclease. HBV DNA of Ba subgenotype can be digested with *Stu* I restriction endonuclease, and HBV DNA of Bj subgenotype can be digested with *Hpa* I restriction endonuclease. Digestion products were subjected to electrophoresis on 2.5% agarose gels.

Determination of HBV BCP mutation

PCR reaction was performed in a total reaction volume of 20 µl.⁶ The reaction conditions for the first PCR were as follows: predenaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 25 s and extension at 72°C for 25 s, and a final extension at 72°C for 5 min. PCR

products (2 µl) were subjected to semi-nested PCR and the reaction conditions were identical to those mentioned above. The reaction products were digested with *Bcl* I restriction endonuclease and the resultant products (10 µl) were subjected to electrophoresis on 2.5% agarose gels. A gel image analysis system was applied to capture the images and the spectrum was used for genotyping.

Determination of HBV pre-C mutation

PCR reaction was performed in a total reaction volume of 20 µl.⁵ The reaction conditions for the first PCR were as follows: predenaturation at 94°C for 1 min, followed by 35 cycles of denaturation at 94°C for 20 s, annealing at 55°C for 25 s and extension at 72°C for 45 s, and a final extension at 72°C for 5 min. The PCR products (2 µl) were subjected to semi-nested PCR and the reaction conditions for the semi-nested PCR were as follows: predenaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 20 s, followed by annealing at 55°C for 20 s and extension at 72°C for 25 s, and a final extension at 72°C for 5 min. The PCR products were digested with *Bsu* 36 I restriction endonuclease and the digestion products (10 µl) were subjected to electrophoresis on 2.5% agarose gels. A gel image analysis system was applied to capture the images and the spectrum was used for genotyping.

Statistical analysis

Statistical analysis was carried out with SPSS version 17.0 and rates comparisons were done with the Chi square test.

RESULTS

Distribution of HBV genotypes and subgenotypes

All 222 patients with chronic HBV infection had HBV genotypes B or C. A total of 93 patients (41.89%) were infected with HBV genotype B and 129 patients (58.11%) with HBV genotype C. Among the 62 patients with HCC, 18 patients (29.03%) were infected with HBV genotype B and 44 patients (70.97%) with HBV genotype C. Of the 70 patients with liver cirrhosis (LC), 34 patients (48.57%) were infected with HBV genotype B and 36 patients (51.43%) with HBV genotype C. Of the 90 patients with CHB, 41 patients (45.56%) were infected with HBV genotype B and 49 patients (54.44%) with HBV genotype C. Statistical analysis showed that a significantly higher number of HCC patients were infected with

HBV genotype C compared to LC patients and CHB patients ($\chi^2=5.258$; 4.220, $P < 0.05$). However, there was no marked difference in the distribution of HBV genotypes between LC patients and CHB patients ($\chi^2 = 0.144$, $P > 0.05$). Ninety-three patients infected with HBV genotype B had the Ba subgenotype. Among the 129 patients infected with HBV genotype C, 88 patients (68.22%) were with C1 subgenotype, while 41 patients (31.78%) with C2 subgenotype. There was no significant difference among the three groups in the distribution of HBV subgenotypes ($\chi^2 = 1.983$, $P > 0.05$).

Correlation of HBV pre-C and BCP mutation with HBV DNA genotype

Among the 222 patients with chronic HBV infection, 74 patients (33.33%) had pre-C mutation, 24 of whom had HBV genotype B (32.43%) and 50 had HBV genotype C (67.57%) ($\chi^2 = 4.080$, $P = 0.045$). In addition, 116 patients had BCP mutation, 32 of whom had HBV genotype B (27.59%) and 84 had HBV genotype C (72.41%) ($\chi^2 = 20.425$, $P = 0.000$). These data suggested that the proportion of patients with BCP mutation was significantly higher in HBV genotype C group compared to HBV genotype B group.

Pre-C and BCP mutation in HCC, LC and CHB patients

Among the HCC patients, LC patients and CHB patients, the proportion of patients with BCP mutation was higher in patients with HBV genotype C compared to patients with HBV genotype B ($\chi^2 = 6.173$, $P < 0.05$). More HCC patients with HBV genotype B presented with the pre-C mutation compared to patients with HBV genotype C ($\chi^2 = 4.555$, $P < 0.05$). The proportion of patients with pre-C mutation was comparable in the LC and CHB groups ($P > 0.05$).

Comparison of HBV gene mutations among the three groups showed that the incidence of pre-C mutation increased with the progression of disease. The HCC, LC, and CHB groups had significantly different proportions of patients with the pre-C mutation (59.68%, 31.43% and 16.67%, respectively; $\chi^2 = 30.727$; $P = 0.000$). The incidence of BCP mutation also increased with the progression of disease: the proportion of patients with BCP mutation in the HCC group, LC group, and CHB group were significantly different (74.19%, 51.43% and 37.78%,

Table 1. Analysis of HBV gene mutations and genotypes in HCC, LC and CHB patients

Groups	pre-C mutation (+)				BCP mutation (+)			
	Type B	Type C	χ^2	<i>P</i>	Type B	Type C	χ^2	<i>P</i>
HCC	7	30	28.56	< 0.01	10	36	15.45	< 0.01
LC	10	12	1.04	> 0.05	13	23	11.63	< 0.01
CHB	7	8	3.57	> 0.05	9	25	13.21	< 0.01
Total	24	50			32	84		

Notes: BCP, basal core promoter of HBV; HCC, hepatocellular carcinoma; LC, post-hepatic cirrhosis; CHB, chronic hepatitis B.

Table 2. Correlation of HBV gene mutations and subgenotypes

Subgenotypes	n	Pre-C mutation (+)	BCP mutation (+)
C1	88	39	61
C2	41	11	23
Ba	93	24	32
χ^2		7.93	22.38
<i>P</i>		< 0.05	< 0.01

Notes: BCP, basal core promoter of HBV.

respectively; $\chi^2 = 19.540$; $P = 0.000$) (Table 1).

Correlation of pre-C mutation and BCP mutation with subgenotypes

The distribution of patients with pre-C mutation or BCP mutation in patients with HBV of different subgenotypes is shown in Table 2. The results showed a marked difference in the proportion of patients with pre-C mutation among patients with different subgenotypes ($\chi^2 = 7.93$, $P < 0.05$). The incidence of pre-C mutation was the highest in patients with subgenotype C1 (44.32%), and the lowest in those with subgenotype Ba (25.81%). The incidence of BCP mutation was the highest in patients with subgenotype C1 (69.32%) and the lowest in those with subgenotype Ba (34.41%) ($\chi^2 = 22.38$, $P < 0.01$).

DISCUSSION

Hepatitis B virus (HBV) has been classified into nine genotypes (A-L) based on inter-genotypic divergence of at least 8% in the complete nucleotide sequence or more than 4% in the S gene.⁷⁻⁸ Each genotype can be classified into different subgenotypes, which have distinct geographical distributions. Our findings were consistent with previous reports showing that HBV genotypes B and C are mainly distributed in Asia, and genotype C is predominant in North China. Although controversial, there is increasing evidence suggesting that HBV genotypes are closely related to the occurrence and development of HCC.⁹ A study evaluating 343 patients from Hong Kong showed

that the distributions of genotype B and C were comparable between HCC patients and non-HCC patients.¹⁰ However, another study from Hong Kong showed that hepatic cirrhosis and HBV genotype C were independent predictors of the occurrence of HCC, and the relative risk was 10.24 and 2.84, respectively after adjustment.¹¹ In present study, HBV genotypes in CHB, LC and HCC patients were investigated. Our results showed a significantly higher incidence of genotype C in HCC patients compared to CHB patients and LC patients, suggesting that genotype C is closely related to the occurrence of HCC. These data are consistent with a previous study from Taiwan.¹² The discrepancy in the distribution of genotype B and C may be explained by the fact that viral load reduction or viral clearance requires a longer immune clearance phase for HBV genotype C compared with HBV genotype B.¹³ Patients with genotype C therefore carry HBV for a longer time. The virus carrier state in HCC is characterized by integrated viral molecules. Several HBV DNA fragments integrated in host chromosomes may cause chromosomal rearrangements and cellular functional disturbance, which result in a vicious cycle of chromosome deletion and rearrangement, leading to the activation of proto-oncogenes, abnormal differentiation of cells and carcinogenesis.¹⁴

GA substitution is the clinically most important mutation in HBV pre-core gene, which may alter the TGG into a stop codon TAG, and result in termination of the translation of pre-C protein and subsequent escape of HBV from immune destruction. However,

this mutation has no influence on viral replication, and the disease is still active. These patients are usually negative for HBeAg. In present study, it showed that HCC patients had a significantly increased incidence of pre-C and BCP mutations, implying that these mutations are related to HCC. In addition, it was showed that the incidence of pre-C and BCP mutations increased with the progression of liver disease: the incidence of pre-C mutation in CHB, LC, and HCC patients was 16.67%, 31.43% and 62.90%, respectively, and the incidence of BCP mutation was 37.78%, 55.71% and 70.97%, respectively ($P < 0.01$). These findings are consistent with a previous study by Zhou et al.¹⁴

Our results showed that genotype C, pre-C mutation and BCP mutation of HBV are all risk factors to HCC, which differed from other recent findings. Yuen et al showed that although BCP mutation not genotype C was closely related to HCC, which may be attributed to the high incidence of BCP mutation in patients infected with HBV genotype C.¹⁵ This finding was similar to the results by Liu et al.¹⁶ The cause of discrepancy among the above findings and our results is still unclear, and we suggest that difference in study populations may be one of the causes. More multicenter population studies are required to confirm our findings.

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