Hepatitis B virus genotypes, phylogeny and occult infection in a region with a high incidence of hepatocellular carcinoma in China

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INTRODUCTION

Infection with hepatitis B virus (HBV) may lead to a wide spectrum of liver diseases ranging from mild, self-limited to fulminant hepatitis in acute infection, and from an asymptomatic carrier state to severe chronic hepatitis, cirrhosis, and hepatocellular carcinoma (HCC) in chronic infection. Human HBV, a prototype member of the family hepadnaviridae, is a circular, partially double-stranded DNA virus of approximately 3200 nt[1]. Traditionally, HBV was classified into 4 subtypes or serotypes (adr, adw, ayr, and ayw) based on antigenic determinants of hepatitis B surface antigen (HBsAg)[2]. Epidemiological studies found that the prevalence of these serotypes varied in different parts of the world. In addition, antibody to the common determinant “a” confers protection against all serotypes. Advances in molecular biology techniques revealed significant diversities in sequences of HBV isolates, accounting for allelic differences among the 4 major HBV serotypes. Based on an intergroup divergence of 8% or more in the complete nucleotide sequence, HBV has been classified into eight genotypes, designated as A to H[3-5]. Recent reports suggested that infections with HBV genotype C were associated with more severe liver diseases, including HCC, than infections with genotype B[6-8]. However, HBV genotype B was suggested to be associated with the development of HCC in Taiwanese below the age of 50 years[9].

Occult HBV infection is characterized by the presence of HBV infection with undetectable HBsAg. Undoubtedly, carriers of occult HBV may transmit the virus through blood transfusion or organ transplantation. Epidemiological and molecular studies performed since the 1980s indicate that persistent HBV infection might play a critical role in the development of HCC and in HBsAg-negative patients[10,11].

The incidence rate of HCC in Long An County, southern Guangxi, China, is about 49.9/100 000, the highest in the world, and over 90% of HCC cases in the county are individuals with positive HBsAg in serum[12,13]. In this study, HBV preC and basic core promoter from 36 HBV asymptomatic carriers in Long An County were amplified and sequenced. The whole genome of a strain from one of the carriers, and genotypes and phylogeny of all the isolates were also analyzed to clarify the difference among HBV strains from different areas. In addition, 52 serum samples from family members of children without hepatitis B vaccination, with negative HBsAg, from the county were

METHODS:

A nested polymerase chain reaction (nPCR) was used for detection of HBV DNA in serum samples from 36 blood donors with asymptomatic HBV infection, and in serum samples from 52 HBsAg negative family members of the children who did not receive hepatitis B vaccination in Long An County. PCR products were sequenced, and the genotype of each HBV sequence was determined by comparison with sequences of known genotypes in the GenBank and EMBL nucleotide databases using the BLAST programme. Phylogenetic trees were constructed by the quartet maximum likelihood analysis using the TreePuzzle software.

RESULTS:

Twenty (55.56%) of 36 HBV asymptomatic carriers were positive for HBV DNA. They were all genotype C by comparison with sequences of known genotypes in the GenBank and EMBL nucleotide databases. The full-length HBV DNA sequence isolated from the sample No. 624 contained 3215 bases. No interesting mutations were found in this isolate. The homology analysis showed that this strain was closer to the Vietnamese HBV genotype C strain, with a homology of 97%, compared its relation to the same genotype of HBV isolated in Shanghai. Six (11.5%) of the 52 HBsAg negative family members were positive for HBV DNA. A point mutation was found in the sample No. 37, resulting in the substitution of amino acid glycine to arginine in the “a” determinant. Other samples with positive HBV DNA did not have any unusual amino acid substitutions in or around the “a” determinant, and were attributed to the wild-type HBV.

CONCLUSION:

The HBVs isolated from asymptomatic carriers of Long An County were all identified as genotype C, and the prevalence of occult HBV infection in the population of the county is as high as 11.5%. It is suggested that genotype C and persistent occult HBV infection may play an important role in the development of HCC in the county.

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detected for HBV DNA to determine the prevalence of occult HBV infection in the population.

**MATERIALS AND METHODS**

**Serum samples**
A total of 36 sera were obtained from asymptomatic blood donors who were infected persistently with HBV in Long An County, southern Guangxi, China. Another 52 serum samples were collected from family members of children failed to have hepatitis B vaccination from the county. Sera were tested for HBsAg, anti-HBc, HBeAg, and anti-HBe using HBV Marker ELISA kits (produced by Xiamen Xinchuang Scientific Technology Company, Limited, Fujian, China) (Tables 1, 2).

**Table 1** Serological markers of 20 asymptomatic carriers with positive HBV DNA

| No. | Age (yr) | Sex   | HBsAg | Anti-HBs | Anti-HBc | HBeAg | Anti-HBe |
|-----|----------|-------|-------|----------|----------|--------|----------|
| 601 | 18       | Male  | +     | -        | -        | +      | -        |
| 602 | 18       | Male  | +     | -        | +        | +      | -        |
| 603 | 28       | Female| +     | -        | +        | +      | -        |
| 604 | 20       | Male  | +     | -        | +        | +      | -        |
| 605 | 27       | Male  | +     | -        | +        | +      | -        |
| 606 | 18       | Male  | +     | -        | +        | +      | -        |
| 607 | 31       | Female| +     | -        | +        | +      | -        |
| 608 | 29       | Female| +     | -        | +        | +      | -        |
| 609 | 28       | Male  | +     | -        | +        | +      | -        |
| 610 | 30       | Male  | +     | -        | +        | +      | -        |
| 611 | 25       | Male  | +     | -        | +        | +      | -        |
| 612 | 24       | Male  | +     | -        | +        | +      | -        |
| 613 | 28       | Male  | +     | -        | +        | +      | -        |
| 614 | 30       | Male  | +     | -        | +        | +      | -        |
| 615 | 38       | Male  | +     | -        | +        | +      | -        |
| 616 | 20       | Male  | +     | -        | +        | +      | -        |
| 617 | 22       | Male  | +     | -        | +        | +      | -        |
| 618 | 19       | Male  | +     | -        | +        | +      | -        |
| 619 | 20       | Male  | +     | -        | +        | +      | -        |
| 620 | 24       | Male  | +     | -        | +        | +      | -        |
| 621 | 28       | Male  | +     | -        | +        | +      | -        |
| 622 | 30       | Male  | +     | -        | +        | +      | -        |
| 623 | 25       | Male  | +     | -        | +        | +      | -        |
| 624 | 28       | Female| +     | -        | +        | +      | -        |
| 625 | 28       | Female| +     | -        | +        | +      | -        |
| 626 | 18       | Male  | +     | -        | +        | +      | -        |
| 627 | 19       | Male  | +     | -        | +        | +      | -        |

**PCR amplification and sequence analysis**
DNA was extracted from 85 μL serum by phenol/chloroform extraction following digestion by pronase. The whole HBV genome was amplified in three fragments. Primers for fragment 1 for the first and second round PCR were LSOB1, BPOLEO1 and LSB11, and POLSEQ2. Primers for fragment 2 for the first and second round PCR were MDN5, BPOLEO1, BPOLBO2 and PSISEQ2. Primers for fragment 3 for the first and second round PCR were POLSEQ1, MDD2, POLSEQ6 and MDC1. The conditions of 30 PCR cycles was at 94 °C for 30 s, at 50 °C for 30 s, and at 72 °C for 90 s in a 50 μL reaction. PCR products from the second round for each fragment were cloned into the vector pCR2.1 (Invitrogen, Leek, The Netherlands) and mini preparations of DNA made from 1.5 mL of 15 μL cultures of individual colonies by phenol extraction and ethanol precipitation of the cell pellet. Plasmids with inserts were identified by digestion with EcoRI and remaining of the cultures used for extraction of DNA using a QIAprep spin kit (Qiagen, Hilden, Germany). The purified DNA was sequenced as above. Sequencing primers for fragment 1 were LSB1, PSISEQ2, 2 s, 2 s, ADELN, MDN4, ADELP, POLSEQ8, POLSEQ9, POLSEQ6, POLSEQ2. Primers for fragment 2 were BPOLEO2, POLSEQ4, LSB11, POLSEQ5 and PSISEQ2. Primers for fragment 3 were POLSEQ6, B935, BPOLEO1, MDN5, B936 and MDC1 (Tables 3, 4).

**Table 3** PCR Primers

| Primers | Sequence | Position (nt) |
|---------|----------|---------------|
| LSB1    | 5'-GGCATATTGTGATCATCCTCTTGGG-3' | 2739-2762    |
| BPOLEO1 | 5'-CTGAGACTCCAAGAGTCTGCTT-3' | 1657-1677    |
| LSB11   | 5'-TTGTCAGCTGCTATTTCTCTT-3' | 2809-2829    |
| POLSEQ2 | 5'-AGCAAAGCTGGGGGATTGCTG-3' | 1168-1188    |
| MDN5    | 5'-GGAGGCTGTAGCATACTTGAT-3' | 1774-1794    |
| BPOLEO1 | 5'-TGCAAGCTCAAGAGGATTGCTG-3' | 1657-1677    |
| BPOLEO2 | 5'-CTTCTTTCTCACATTTGGAAGA-3' | 2216-2236    |
| PSISEQ2 | 5'-CTGAGACGAGGAGGGCTTCTCTG-3' | 85-84       |
| POLSEQ5 | 5'-ACCAACGGTTGGGCTTCTCT-3' | 847-866      |
| MDD2    | 5'-GAAGAATAAGAGGCTTCTAAT-3' | 2481-2500    |
| POLSEQ6 | 5'-TTTCCATTTCTGGGCACTTT-3' | 1089-1109    |
| MDC1    | 5'-GGCATTTGAGGAGGGCGATTTG-3' | 2304-2324    |
The homology with 23 HBV strains in GenBank was determined by nested PCR amplification. Six samples of 52 family members of children without immunization of HBV vaccine were positive for HBV DNA, counting for 11.5% (6/52) (Table 2). The other samples were negative for both HBsAg and anti-HBs. The other samples with positive HBV DNA did not have unusual amino acid substitutions in or around the “a” determinant, and were attributed to the wild-type HBV of genotype C.

**Phylogeny analysis**

The homology with 23 HBV strains in GenBank was determined by using the programme TreePuzzle. The HBV strain isolated from sample No. 624 in Long An County was closer to the Vietnamese HBV of genotype C, with 97% homology between them, as compared to the isolates of the same genotype from Shanghai, Beijing and Tibet in evolution (Figure 1).

**RESULTS**

**HBV genotypes**

Compared with sequences of known genotypes in the GenBank and EMBL nucleotide databases using the programme BLAST[14]. Phylogenetic trees were constructed by maximum likelihood analysis by quartet puzzling[15]. TreePuzzle is available at http://www.tree-puzzle.de.

**Characterization of HBV genome structure**

The full-length HBV-DNA sequence of HBV isolated from sample No. 624 in Long An County was sequenced. A point mutation from guanosine to adenosine at nucleotide position 587 resulted in an amino acid substitution from glycine to arginine in the highly antigenic “a” determinant, and were attributed to the wild-type HBV of genotype C.

**Database sequences**

The following complete genomes (represented by their accession number) were used in phylogenetic tree analyses: genotype A: AJ344115; genotype B: AF282917, D00331; genotype C: AY040627, AF461357, M38454, AY057948, AF241410, AF241411, AF330110, AB026812, D00329, AB014398, X75665, X52939, X59795; genotype D: AF280817, AJ344116; genotype E: X75664; genotype F: AB036908; genotype G: AF405706.

**Genotype and phylogeny analysis**

The genotype of each HBV sequence from the 20 asymptomatic HBV carriers in Long An County were found to be genotype C viruses. There were 11, 2, 3 point mutations in PreS1, PreS2 and S regions. The amino acid mutation in PreS1 was TPP→PHQ at amino acid position 182 in sample No. 624 in Long An County was closer to the Vietnamese HBV of genotype C, with 97% homology between them, as compared to the isolates of the same genotype from Shanghai, Beijing and Tibet in evolution (Figure 1).
the Mediterranean region, while genotype E is found mainly in West Africa. Genotype F shows the highest divergence among the genotypes and is indigenous to aboriginal populations of the Americas[20]. Genotype G was found in USA and France[4], genotype H in the Central America[5], and genotypes A, B, C and D in China. The predominant genotype in China is genotypes B and C[21,22].

The incidence of HCC in Long An County is about 49.9/100,000 and the prevalence of HBsAg in the county is 16%[13,21] and more than 90% of hepatocellular carcinoma (HCC) cases are individuals positive for HBsAg in serum[12]. In Japan, HBV genotype C was found to be closely associated with severe liver diseases and the development of HCC[7,24], although young HCC patients were found to be HBV genotype C in Taiwan[25].

HBV infection is usually diagnosed when the circulating HBsAg is detected. However, advances in molecular biology techniques revealed that a low level of HBV DNA could be detected in serum and liver tissue in some individuals who were negative for HBsAg[26-27]. Although there are some studies on occult HBV infection, the precise prevalence of this clinical entity is still unknown. Luo et al.[28] found that the prevalence rate of occult HBV infection in Guangdong and Hainan Provinces was 2.0% (6/294) and 3.4% (68/1995), respectively in the general population. Bowet et al.[29] reported that the prevalence was 4.3% (11/258) in subjects without liver disease in USA. In this study, we found that the prevalence of occult HBV infection in Long An County was higher (11.5%, 6/52). The mechanisms that HBV carriers have a low, but stable level of viral replication remain to be defined. HBV strains might have mutations in S region resulting in occult HBV infection[60,61]. This kind of mutations was found in one of our samples. In addition, this type of carriers should be added to the typical HBsAg-positive carriers constituting about 16% of the general population, to estimate more precisely the proportion of asymptomatic HBV carriers in Long An County. It is clear that the prevalence of HBV infection in Long An County is in correspondence with its high incidence of HCC.

To extend the knowledge of molecular features of natural HBV isolates, sample No. 624 in this study was selected for whole genome sequencing. This strain contained 3215 bases. HBsAg region sequence showed that it belonged to serotype adw. No special mutation which could change the expression function of viral proteins was found in the sequence of this isolate. By homology analysis with 23 HBV strains in GenBank, this isolate was found to be closer to the Vietnamese HBV genotype C strain[22] than to the genotype C isolates from Shanghai in evolution[30], with 97% homology with the Vietnamese isolate. At present, the only explanation about this is that Long An County is geographically close to Vietnam.

In summary, HBV infections in Long An County are attributable to HBV genotypes C. The prevalence of occult HBV infection in Long An County is higher.

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