Original Article

Prevalence of hepatitis b virus genotype I in Fusui, China

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Abstract: Objectives: The objective of this study was to clarify the genotypes of hepatitis B virus (HBV) recombinants found in Fusui, China. Methods: Ninety-six HBV DNA-positive serum specimens were collected from Fusui County, Guangxi Province, China, in this study. Direct sequencing was conducted for the HBV-nested PCR products. Results: Four A/C/G recombination sequences were detected after genome-wide analysis using the National Center for Biotechnology Information (NCBI) genotyping tool. They were presumed to be a new genotype based on the application of phylogenetic and recombination analysis. The recombination sites were similar. The difference between the recombination sequences and other genotypes accounting for approximately 8% was existed. The Basic Local Alignment Search Tool was run to compare the four sequences in GenBank. Twenty sequences had ≥98% similarity with the recombination sequences. The four recombination sequences were identified as genotype I, followed by a mini review of genotype I. Conclusion: We reported on the presence of genotype I for the first time in Fusui. Genotype I is mainly distributed in Southwest China, Guangxi and Southeast Asian countries, such as Vietnam and Laos, which border China. Consequently, our study has made a valuable contribution to the NCBI genotyping tool.

Keywords: Hepatitis B virus, mutation, recombinant virus

Introduction

The hepatitis B virus (HBV) is a partially double-stranded circular DNA virus, at approximately 3.2 kb, and distributed worldwide. Chronic hepatitis, cirrhosis, and liver cancer are closely related to it. The number of chronically infected people in China accounts for roughly one third of the global number of HBV infections [1]. Based on a ≥8% nucleotide sequence divergence [2, 3], HBV can be divided into eight genotypes (A-H). The geographical distribution of HBV genotype differs. Genotypes B and C account for the majority in China. In recent years, new genotypes and subtypes have been reported [4, 5]. Vietnam and Laos, bordering China, have reported on genotype I [6, 7]. Studies regarding HBV genotype I are limited in China. In the current study, recombinant HBV genotypes were found in Fusui. According to evolutionary analysis and identification, four cases with genotype I were confirmed. A mini literature review was performed to understand the geographical distribution of genotype I.

Materials and methods

Subjects

This study was approved by the Guangxi Medical University Ethics Committee. All of the patients provided informed consent to participate in the study. Between April 2015 and January 2016, a total of 96 patients with HBV DNA positive were included. All subjects were native to Fusui.

PCR and sequencing

HBV DNA was extracted from 200 μl serum samples using viral DNA/RNA out kit (TIANGEN Biotech, Beijing, China). Two fragments of HBV genome were amplified using semi-nested PCR, and were identified as fragments A and B.

First-round PCR for A fragment was conducted with primers P3 and AR1. PCR reaction was carried out in a 50 μl mixture, containing 10 μl 5× Reaction Buffer, 4 μl 2.5 mmol/L deoxynucleoside triphosphates (dNTPs), 2 μl 10 mmol/L
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**Table 1.** HBV genome sequencing primers

<table>
<thead>
<tr>
<th>Primers</th>
<th>5’-3’ nt bp</th>
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<tbody>
<tr>
<td>P3</td>
<td>TTTCACTTCGGGCTACTAC nt 1823-1841 19 bp</td>
</tr>
<tr>
<td>AR1</td>
<td>ACAGTTGAGGAAGACG nt 723-709 15 bp</td>
</tr>
<tr>
<td>AR2</td>
<td>AGAACCGAGCTAGGCA nt 665-650 16 bp</td>
</tr>
<tr>
<td>P4</td>
<td>AAGTTGCAAATGGCTCTG nt 1823-1806 18 bp</td>
</tr>
<tr>
<td>AF1</td>
<td>GTCTGGCGGCTTTATAC nt 381-397 17 bp</td>
</tr>
<tr>
<td>AF2</td>
<td>TGCCCGTTGTGCCTCCTA nt 465-481 17 bp</td>
</tr>
<tr>
<td>WP0</td>
<td>CTCGCGAAAGATCTCAAT nt 2414-2432 19 bp</td>
</tr>
<tr>
<td>WP1</td>
<td>AGGCTTCCGGAGCAAGAGC nt 2024-2004 21 bp</td>
</tr>
<tr>
<td>WP2</td>
<td>TGCACCTCCGCTCCCTGTG nt 1580-1599 20 bp</td>
</tr>
</tbody>
</table>

Primers (sense and antisense), and PrimeSTAR GXL DNA Polymerase (1.25 U/µl) (Takara, Dalian, China).

First-round PCR was performed as follows: 98°C for two minutes, 98°C for 10 seconds, 55°C for 15 seconds, 68°C for two minutes and 30 seconds for 35 cycles, and finally, 68°C for 10 minutes. The first-round PCR product (2 µl) was reamplified under the same PCR conditions as those for the first-round reaction. Second-round PCR for A fragment was conducted with primers P3 and AR2, with a product length of 2058 bp. First-round PCR on B fragment was conducted with primers P4 and AF1. PCR was performed as follows: 98°C for two minutes, 98°C for 10 seconds, 55°C for 15 seconds, 68°C for one minute and 45 seconds for 35 cycles, and finally, 68°C for 10 minutes. The first-round PCR product (2 µl) was reamplified under the same PCR conditions as those for the first-round reaction. Second-round PCR on B fragment was conducted with primers P4 and AF2, with a product length of 1359 bp. The primers used are listed in Table 1.

Second-round PCR product was purified using QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany) and sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and an ABI PRISM 3730 DNA Analyzer (Applied Biosystems, Foster City, USA).

**HBV genotyping**

The sequencing results were processed using LaserGene version 7.1.0 (1989-2006) (DNASTAR, Madison, USA). Initially, the genotypes of the four specimens were identified using the National Center for Biotechnology Information (NCBI) online typing tool (http://www.ncbi.nlm.nih.gov/Projects/genotyping/formpage.cgi).

**Basic local alignment search tool**

The Basic Local Alignment Search Tool (BLAST) was run for the recombination sequences in GenBank. Twenty sequences which were submitted by six research groups had a similarity of ≥98% with recombination sequences.

**Phylogenetic analyses**

Sequence alignment was carried out using Clustal X version 2.0 (Bioinformatics, 2007) software. Phylogenetic trees were created by the neighbor-joining method using Molecular Evolutionary Genetics Analysis (Mega) version 6.0 software (Tamura, Stecher, Peterson, Filipski, and Kumar, 2013) by which bootstrap analysis was performed in 1000 trial replications. Genetic distance was calculated and pairwise distance was compared using Kimura’s two-parameter model (Mega). The different subtypes of HBV genomes were included in the analysis (Figure 1).

**Recombination analysis**

Complex recombination between genotypes A/C/G in isolates 414, 441, 533, 678 was observed. The four strains were subjected to bootscan analysis over the complete genome, using the SimPlot 3.5 program (http://sray.med.som.jhmi.edu/SCRoftware/simplot/) with 200-bp window size, 20-bp step size, and 100 bootstrap replicates, with gap-stripped alignment and neighbor-joining analysis. The strains were compared with three representative HBV genotypes: A (accession no. AB126580), C (accession no. AB049609), and G (accession no. AB056513). The vertical lines showed the common estimated breakpoints of recombination in all of the analyzed strains (Figure 2).

**Pre S/S gene analysis**

The amino acid sequences deduced by pre S/S gene were analyzed and compared, including the reference sequences for genotypes A/C/G, the two sequences from the current study, and similar sequences obtained in BLAST (Figure 3).

**Complete genome analysis**

Whole-genome sequencing was performed. The nucleotide differences between the four
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**Results**

There were four specimens (414, 441, 533, 678) preliminarily classified as the A/C/G recombination using the NCBI online typing tool. BLAST was run to compare these sequences. There were twenty sequences submitted by six research groups with ≥98% similarity, most of which were classified as genotype I. Phylogenetic tree analysis indicated that these sequences did not belong to any of the other genotypes. The recombination analysis demonstrated that these sequences had similar recombination sites, requiring to define as a new category, rather than an occasional recombinant gene type. Besides, there was no relationship between the four patients.

Conservative amino acids, such as 56 histidine, 60 alanine, 87 asparagine, 90 valine, 91 valine, 136 isoleucine, 166 threonine, 198

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Figure 1. The phylogenetic tree analysis of 4 recombinants and NCBI reference sequences. *Scale bars represent the number of substitutions per site.*

- Variance estimation method: The bootstrap method.
- Number of bootstrap replications: 1000.
- Model: Kimura’s two-parameter model.
- Gap data treatment: Pairwise deletion.

The remaining options were set as defaults. Subsequently, the difference rate and standard deviation of the four sequences were calculated after a pairwise comparison of the sequences (Table 2). All strains in the new clade had Ile110, Thr126, and Lys160 in the S gene, and were identified as serotype adw.
Figure 2. Recombination analysis of A/C/G recombinants.
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<table>
<thead>
<tr>
<th>Accession</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AJ8125580</td>
<td>1 MGGWS SKPRK GMSN PLGFSPDHQL DPAFGANSN PDWFDNPIKD HWPAANQVGV 60</td>
</tr>
<tr>
<td>AJ8125580</td>
<td>61 GAGFPGGLTPP HGGILGWSPQ AQGILTTVST IPPASTNRQ SGRQPTISP PLDRDRHPQAK 120</td>
</tr>
<tr>
<td>AJ8056513</td>
<td>61 YFLLPAI S .L .N .PVV . 120</td>
</tr>
<tr>
<td>AJ8231908</td>
<td>61 YFLLPAI S .L .N .PVV . 120</td>
</tr>
<tr>
<td>AJ8241407</td>
<td>61 YFLLPAI S .L .N .PVV . 120</td>
</tr>
<tr>
<td>fs414</td>
<td>61 YFLLPAI S .L .N .PVV . 120</td>
</tr>
<tr>
<td>fs678</td>
<td>61 YFLLPAI S .L .N .PVV . 120</td>
</tr>
<tr>
<td>AJ8125580</td>
<td>121 QWNSTAFHQA LQDPVRGLY FPAGGSSSGT VNPAPEHISLISIARTGD PVTMNPIETS 180</td>
</tr>
<tr>
<td>AJ8125580</td>
<td>181 QFLQPLLLVLQ AGFLLTLTRIL TIPOLIDSWWTSSLNFLGGSP VCLGQNSQSP TSNHSPSCP 240</td>
</tr>
<tr>
<td>AJ8125580</td>
<td>241 PICPGYRWMC LRRFIIFLFIL LLLLCLIFLLLVLDDYQGLMPV CPLIPSGTSTT STGPCKTCTT 300</td>
</tr>
<tr>
<td>AJ8125580</td>
<td>301 PAQGNSMFPS CCGTKPSNGNCCTCI PI PSSW AFAKYLWEEWA SVRFSWLSLVLPPVQVFWG 360</td>
</tr>
<tr>
<td>AJ8056513</td>
<td>301 T .S .RF . 360</td>
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<td>301 T .S .RF . 360</td>
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<tr>
<td>fs678</td>
<td>301 T .S .RF . 360</td>
</tr>
<tr>
<td>AJ8125580</td>
<td>361 SPTVWL SAIW MMWYWGSPSLY SI VNPCPPLL PI FFCWVYI * 400</td>
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<tr>
<td>AJ8056513</td>
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<tr>
<td>fs678</td>
<td>361 T .S .RF . 400</td>
</tr>
</tbody>
</table>
GenBank. Unique conservative amino acids were discovered, such as 56 histidine, 60 alanine, 87 asparagine, 90 valine, 91 valine, 136 isoleucine, 166 threonine, 198 lysine, and 342 valine, which didn’t belong to genotypes A/C/G. Nine unique amino acids in the conserved region would have an important effect on protein expression. These findings support the decision to divide the HBV sequence into a new genotype.

Following the literature review, we found out that Charles [9], a Swedish scientist, originally found similar sequences in Hanoi, Vietnam, and submitted three HBV sequences to GenBank. Later in 2008, Huy amplified the HBV genomes using single-round of amplification [6]. One of the specimens also came from Hanoi and was found to be a recombinant of A/C/G. After the evolution analysis, recombination analysis and amino acid sequence analysis, the one sequence was presumed to be a new genotype I. In the same year, Olinger [7] reported 19 cases of genotype I from 389 cases of HBV-carrying blood donors in Laos, Vientiane. Fang [10] reported similar recombinant genotypes in a 10-year cohort study in Longan County, Guangxi, China, which accounted for 13.5% (38/281) of all genotypes in 2011. In 2013, they classified the genotype as I and speculated that it originated in Longan County, Guangxi, China [11]. Additionally, Two specimens from people of Yi nationality in Sichuan Province were found to be infected by genotype I in 2012 [12]. In 2014, Su [13] reported that 2 out of 276 cases of chronic hepatitis were A/C/G recombination in Guilin, Guangxi, China. We presumed that they were genotype I, based on the sequences provided. Of the 2 cases of genotype I from 72 patients with chronic hepatitis B were also discovered in Yunnan Province, China [14].

On the basis of the findings in our study, We suggested that the previously reported HBV genomes of the A/C/G recombinants mainly distributed in Guangxi [11], Shanxi [4], Yunnan [15], and Sichuan [12] in China.

Conclusions
According to the results of our study, we believe that whole HBV genome differences and different amino acids in the pre S/S gene meet the
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classification standards for a new genotype. Meanwhile, the A/C/G recombination sequenc- 
eses should be classified as HBV genotype I which mainly exists in Guangxi Province, Southwest of 
China, and can also be found in other Southeast 
Asian countries, such as Vietnam and Laos, 
bordering China. Accordingly, our study has 
made a valuable contribution to the NCBI geno-
typing tool.

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Disclosure of conflict of interest

None.

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