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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

Table 1. HBV genome sequencing primers

	5'-3'	nt	bp
P3	TTTCACCTCTGCCTAATCA	nt 1823-1841	19 bp
AR1	ACAGTGGGGGAAAGC	nt 723-709	15 bp
AR2	AGAAACGGACTGAGGC	nt 665-650	16 bp
P4	AAAGTTGCATGGTGCTGG	nt 1823-1806	18 bp
AF1	GTCTGCGGCGTTTTATC	nt 381-397	17 bp
AF2	TGCCCGTTTGTCCTCTA	nt 465-481	17 bp
WP0	CGTCGCAGAAGATCTCAAT	nt 2414-2432	19 bp
WP1	AGGCCTCCCGATACAGAGCAG	nt 2024-2004	21 bp
WP2	TGCACTTCGCTTCACCTCTG	nt 1580-1599	20 bp

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 were 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 analized 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

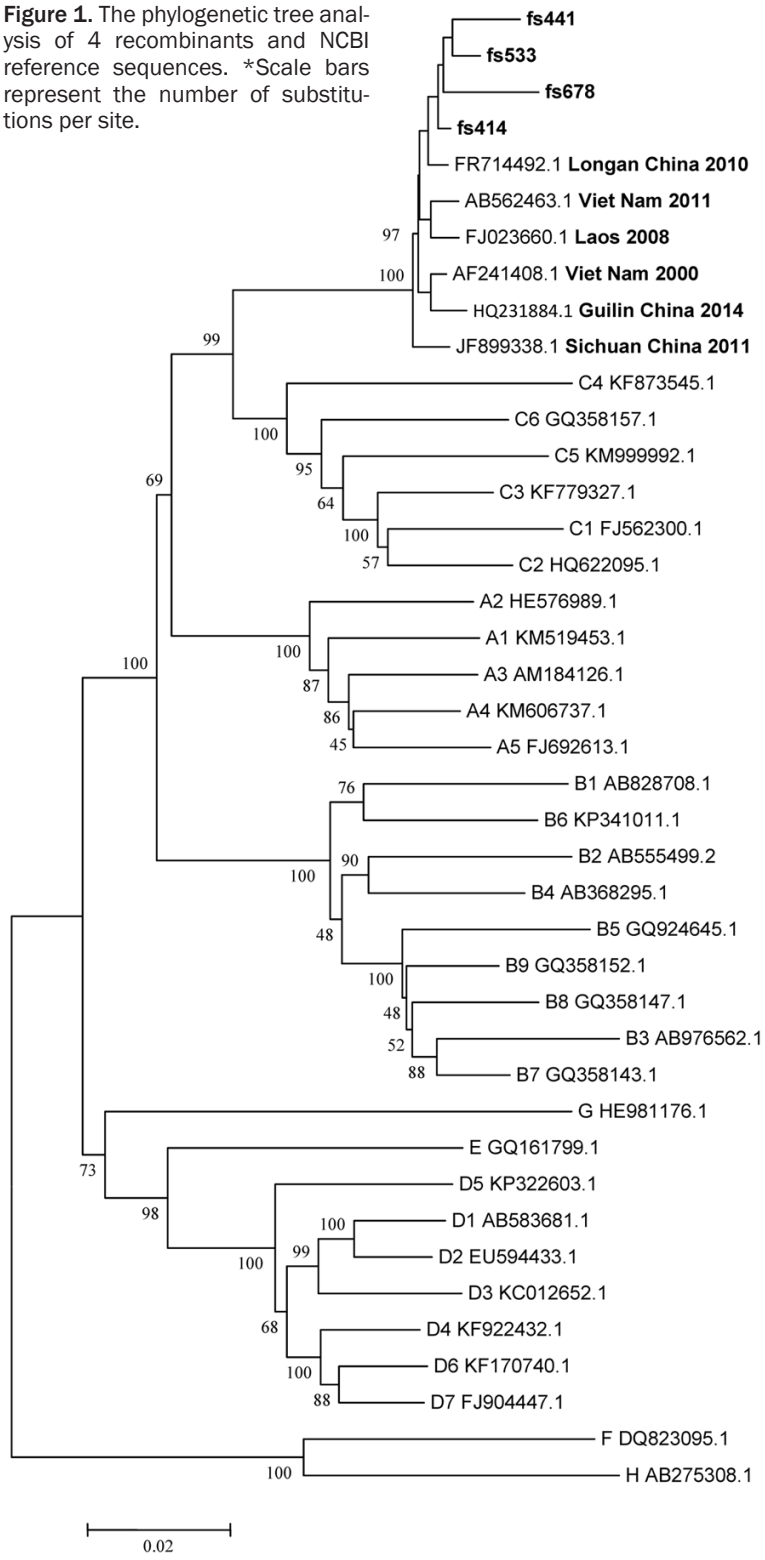

Figure 1. The phylogenetic tree analysis of 4 recombinants and NCBI reference sequences. *Scale bars represent the number of substitutions per site.

recombinants and the A/C/G reference sequences were compared using Mega. The parameters were set as follows:

•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.

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 as 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

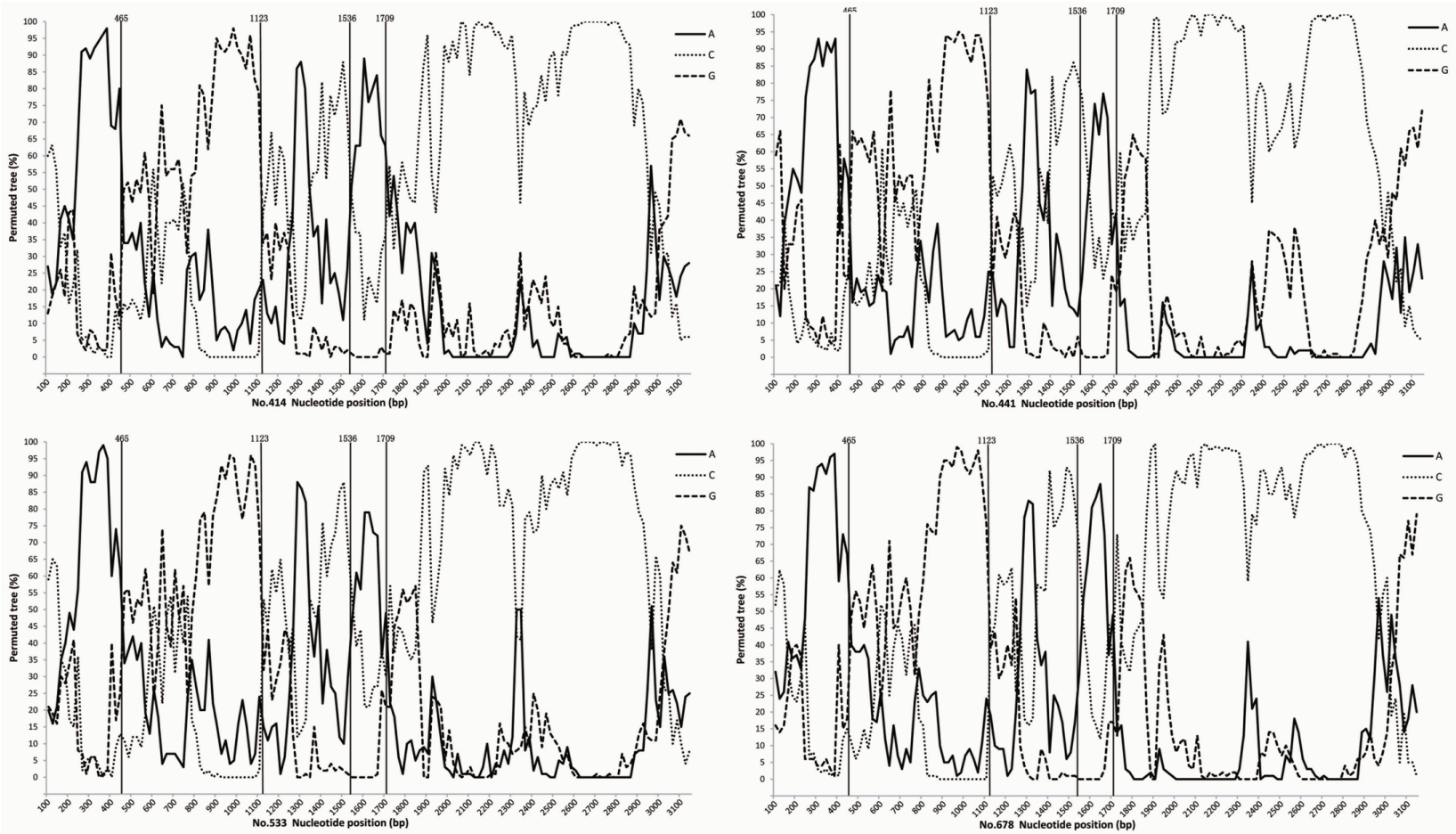

Figure 2. Recombination analysis of A/C/G recombinants.

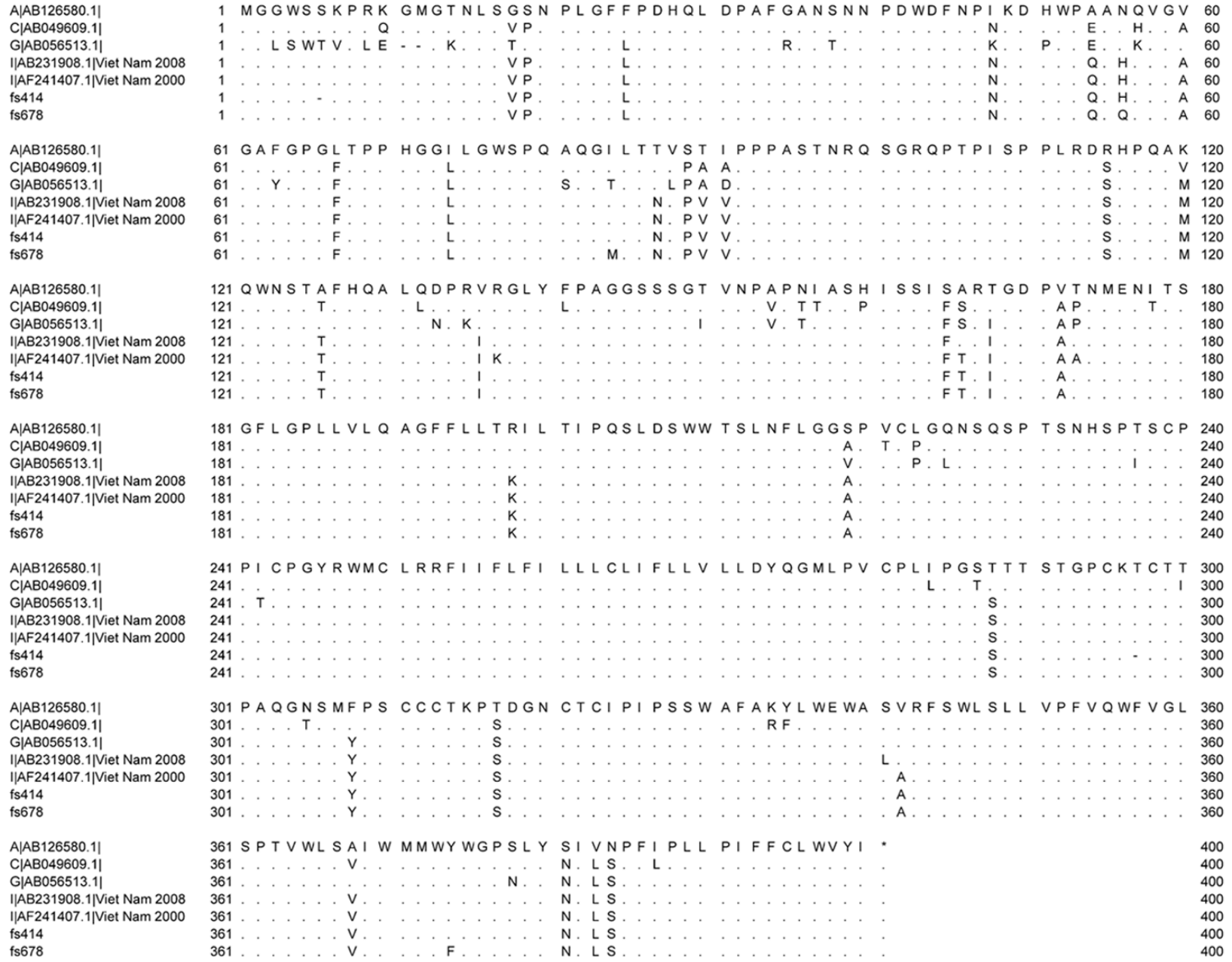
A|AB126580.1| 1 MGGWSSKPRK GMGTNLSGSN PLGFFPDHQL DPAFGANSNN PDWDFNPIKD HWPAANQVGV 60
C|AB049609.1| 1 .....Q..... ..VP..... ..........N....E..H..A 60
G|AB056513.1| 1 ..LSWTV.LE--K...T..... ....L.....R..T.....K..P..E..K... 60
I|AB231908.1|Viet Nam 2008 1 ....VP..... ....L..... ..........N....Q.H...A 60
I|AF241407.1|Viet Nam 2000 1 ....VP..... ....L..... ..........N....Q.H...A 60
fs414 1 .....-..... ..VP..... ....L..... ..........N....Q.H...A 60
fs678 1 ....VP..... ....L..... ..........N....Q.Q...A 60
A|AB126580.1| 61 GAFGPGLTPP HGGILGWSPQ AQGILTTVST IPPPASTNRQ SGRQPTPISP PLRDRHPQAK 120
C|AB049609.1| 61 .....F..... ..L..... ....PA A..... ....S....V 120
G|AB056513.1| 61 ..Y...F..... ..L..... S..T...LPA D..... ....S....M 120
I|AB231908.1|Viet Nam 2008 61 .....F..... ..L..... ....N.PV V..... ....S....M 120
I|AF241407.1|Viet Nam 2000 61 .....F..... ..L..... ....N.PV V..... ....S....M 120
fs414 61 .....F..... ..L..... ....N.PV V..... ....S....M 120
fs678 61 .....F..... ..L..... ....M...N.PV V..... ....S....M 120
A|AB126580.1| 121 QWNSTAFHQA LQDPRVRGLY FPAGGSSSGT VNPAPNIASH ISSISARTGD PVTNMENITS 180
C|AB049609.1| 121 .....T..... .L..... ..L..... ..........V.TT..P.... FS..... ..AP.....T.. 180
G|AB056513.1| 121 ....N.K..... ....I... V.T..... ..FS.I... ..AP..... 180
I|AB231908.1|Viet Nam 2008 121 .....T..... ...I..... ..........F..I... ..A..... 180
I|AF241407.1|Viet Nam 2000 121 .....T..... ...IK..... ..........FT.I... ..AA..... 180
fs414 121 .....T..... ...I..... ..........FT.I... ..A..... 180
fs678 121 .....T..... ...I..... ..........FT.I... ..A..... 180
A|AB126580.1| 181 GFLGPLLVLQ AGFFLLTRIL TIPQSLDSWW TSLNFLGGSP VCLGQNSQSP TSNHSPTSCP 240
C|AB049609.1| 181 ....A..T.P..... 240
G|AB056513.1| 181 ....V...P.L..... .....I... 240
I|AB231908.1|Viet Nam 2008 181 ....K..... ....A..... 240
I|AF241407.1|Viet Nam 2000 181 ....K..... ....A..... 240
fs414 181 ....K..... ....A..... 240
fs678 181 ....K..... ....A..... 240
A|AB126580.1| 241 PICPGYRWMC LRRFIIFLFI LLLCLIFLLV LLDYQGMLPV CPLIPGSTTT STGPCKTCTT 300
C|AB049609.1| 241 ....L..T..... .....I 300
G|AB056513.1| 241 T..... ....S..... 300
I|AB231908.1|Viet Nam 2008 241 ....S..... 300
I|AF241407.1|Viet Nam 2000 241 ....S..... 300
fs414 241 ....S.....-... 300
fs678 241 ....S..... 300
A|AB126580.1| 301 PAQGNSMFPS CCCTKPTDGN CTCIPIPSSW AFAKYLWEWA SVRFSWLSLL VPFVQWFVGL 360
C|AB049609.1| 301 ....T..... ..S..... ....RF..... 360
G|AB056513.1| 301 .....Y..... ..S..... 360
I|AB231908.1|Viet Nam 2008 301 .....Y..... ..S..... ..... .L..... 360
I|AF241407.1|Viet Nam 2000 301 .....Y..... ..S..... ..... .A..... 360
fs414 301 .....Y..... ..S..... ..... .A..... 360
fs678 301 .....Y..... ..S..... ..... .A..... 360
A|AB126580.1| 361 SPTVWLSAIW MMWYWGPSLY SIVNPFIPLL PIFFCLWVYI * 400
C|AB049609.1| 361 .....V..... ....NLS..L..... 400
G|AB056513.1| 361 ....N..NLS..... 400
I|AB231908.1|Viet Nam 2008 361 .....V..... ....NLS..... 400
I|AF241407.1|Viet Nam 2000 361 .....V..... ....NLS..... 400
fs414 361 .....V..... ....NLS..... 400
fs678 361 .....V.....F..... ..NLS..... 400

Figure 3. Comparison of amino acids in Pre S and S region of recombinant specimens and reference sequences.

Table 2. Nucleotide distances between the Fusui HBV recombinants and other reference genotype strains (mean ± SD)

No.	Reference sequences		
	A	C	G
414	8.2±0.5	7.9±0.5	11.6±0.6
441	9.0±0.5	8.4±0.5	11.6±0.6
533	8.0±0.5	8.0±0.5	11.7±0.6
678	9.3±0.5	8.9±0.6	12.5±0.6

lysine, and 342 valine, were identified by pre S/S gene analysis. It was found that they didn't belong to genotypes A/C/G. The most similar genotype identified was C with nucleotide sequence divergence of roughly 8%. Nucleotide sequence divergence increased to ≥8% when a comparison was made with genotypes A or G. Taking the differences that were identified by the pre S/S gene analysis into account, we classified the four recombination sequences as genotype I.

Discussion

Full-length sequence and functional analysis of HBV were obtained by direct sequencing of the two fragments in our study, which effectively reduced artificial recombination and greatly improved detection sensitivity. A series of bioinformatic methods were adopted in our study for classifying the genotype and subtype of these samples.

The recombination sequences were compared with the reference sequences of genotype A/C/G. The most similar genotype was C, with nucleotide sequence divergence of 7.9-8.9%. Nucleotide sequence divergence in relation to the other types was ≥8%. They meet the criteria of a new genotype [8]. The result of recombination analysis showed that nt 1709-3000 was basically consistent with type C. In addition, type G sequences were identified in the Pre S/S gene. The nt 150-465 region was more similar to genotype A, while nt 465-1123 was more similar to genotype G.

In order to confirm whether the recombination genotypes could be classified as genotype C, the corresponding amino acids in the pre S/S region were compared with the reference sequences and BLAST was carried out in 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

classification standards for a new genotype. Meanwhile, the A/C/G recombination sequences 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 genotyping tool.

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Disclosure of conflict of interest

None.

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References

[1] Schweitzer A, Horn J, Mikolajczyk RT, Krause G and Ott JJ. Estimations of worldwide prevalence of chronic hepatitis B virus infection: a systematic review of data published between 1965 and 2013. Lancet 2015; 386: 1546-1555.

[2] Kramvis A, Kew M and Francois G. Hepatitis B virus genotypes. Vaccine 2005; 23: 2409-2423.

[3] Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, Robertson BH, Locarnini S and Magnius LO. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004; 47: 289-309.

[4] Yu H, Yuan Q, Ge SX, Wang HY, Zhang YL, Chen QR, Zhang J, Chen PJ and Xia NS. Molecular and phylogenetic analyses suggest an additional hepatitis B virus genotype "I". PLoS One 2010; 5: e9297.

[5] Tatematsu K, Tanaka Y, Kurbanov F, Sugauchi F, Mano S, Maeshiro T, Nakayoshi T, Wakuta M, Miyakawa Y and Mizokami M. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J Virol 2009; 83: 10538-10547.

[6] Tran TT, Trinh TN and Abe K. New complex recombinant genotype of hepatitis B virus identified in Vietnam. J Virol 2008; 82: 5657-5663.

[7] Olinger CM, Jutavijittum P, Hubschen JM, Yousukh A, Samountry B, Thammavong T, Toriyama K and Muller CP. Possible new hepatitis B virus genotype, southeast Asia. Emerg Infect Dis 2008; 14: 1777-1780.

[8] Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y and Mayumi M. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988; 69: 2575-2583.

[9] Hannoun C, Norder H and Lindh M. An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. J Gen Virol 2000; 81: 2267-2272.

[10] Fang ZL, Hue S, Sabin CA, Li GJ, Yang JY, Chen QY, Fang KX, Huang J, Wang XY and Harrison TJ. A complex hepatitis B virus (X/C) recombinant is common in Longan county, Guangxi and may have originated in southern China. J Gen Virol 2011; 92: 402-411.

[11] Li GJ, Hue S, Harrison TJ, Yang JY, Chen QY, Wang XY and Fang ZL. Hepatitis B virus candidate subgenotype I1 varies in distribution throughout Guangxi, China and may have originated in Longan county, Guangxi. J Med Virol 2013; 85: 799-807.

[12] Tong W, He J, Sun L, He S and Qi Q. Hepatitis B virus with a proposed genotype I was found in Sichuan province, China. J Med Virol 2012; 84: 866-870.

[13] Su H, Liu Y, Xu Z, Cheng S, Ye H, Xu Q, Liu Q, Tan S, Xu D and Liu Y. A novel complex A/C/G intergenotypic recombinant of hepatitis B virus isolated in southern China. PLoS One 2014; 9: e84005.

[14] Wang B, Feng Y, Li Z, Duan H, Zhao T, Zhang A, Liu L, Baloch Z and Xia X. Distribution and diversity of hepatitis B virus genotypes in Yunnan, China. J Med Virol 2014; 86: 1675-1682.

[15] Kang WY, Bi SL, Ding ZR, Tian BJ, Zhao ZX and Li H. [Molecular identification of hepatitis B virus genotype I in Yunnan province, the People's Republic of China]. Bing Du Xue Bao 2011; 27: 215-217.