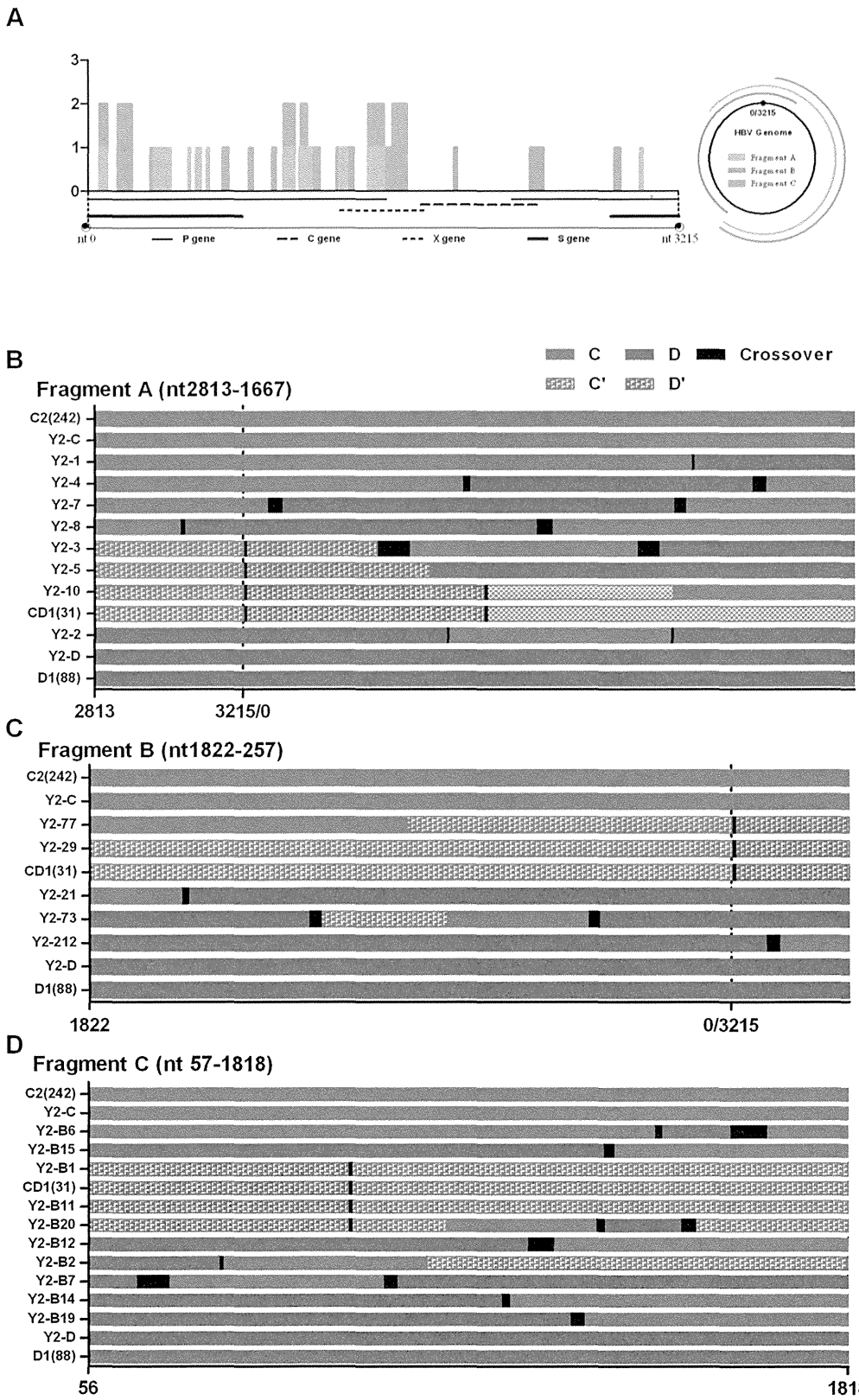

A
3
2
1
0
nt 0
P gene
C gene
X gene
S gene
nt 3215
0/3215
HBV Genome
Fragment A
Fragment B
Fragment C
B
C
D
Crossover
C'
D'
Fragment A (nt2813-1667)
C2(242)
Y2-C
Y2-1
Y2-4
Y2-7
Y2-8
Y2-3
Y2-5
Y2-10
CD1(31)
Y2-2
Y2-D
D1(88)
2813
3215/0
Fragment B (nt1822-257)
C2(242)
Y2-C
Y2-77
Y2-29
CD1(31)
Y2-21
Y2-73
Y2-212
Y2-D
D1(88)
1822
0/3215
Fragment C (nt 57-1818)
C2(242)
Y2-C
Y2-B6
Y2-B15
Y2-B1
CD1(31)
Y2-B11
Y2-B20
Y2-B12
Y2-B2
Y2-B7
Y2-B14
Y2-B19
Y2-D
D1(88)
56
1818

Figure 3. Alignment and recombination crossover regions found in Y2 clones. (**A**) Frequency and distribution of the recombination crossover regions found in Y2 clones along the HBV genome. The bars indicate the number of clones (y axis) showing recombination crossover regions at each site. The 1-3215 of x axis was consistent with the nt1-3215 of HBV genome. Different colors represent the sites find from clones of different PCR region: pink bars for fragment A, grey bars for fragment B and green bars for fragment C. (**B**) Alignment of fragment A (HBV nt 2813-0-1667). Y2-1′12: clones from fragment A of Y2 patients. (**C**) Alignment of fragment B (HBV nt 1822-0-257). Y2-21′212: clones from fragment B of Y2 patients. (**D**) Alignment of fragment C (HBV nt 57-1818) of Y2 clones. Y2-B1′B22: clones from fragment C of Y2 patients. The number on the x axis was consistent with the site of nucleotides of HBV genome. Solid green lines are genotype C2, solid pink lines are genotype D1, speckled green lines are the C2 component of genotype recombinant CD1 and speckled pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242) is the consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88) is the consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33) is the consensus sequence formed by CD1 recombinant sequences from GenBank.
doi:10.1371/journal.pone.0038241.g003

suggests C2 and D1 are parental sequences of CD1 and CD2 recombinants. Virological differences among HBV genotypes were demonstrated in vitroand in CHiM mice, with genotype C having a higher replication capacity than D [23]. Why does the replication-deficient genotype D virus predominate over replication-competent genotype C? As mixed HBV infections together with recombination are rare, we have little knowledge about i this situation. On the one hand, we know little about host impact on different genotypes and recombinants. On the other hand, we know little about interference and competition in the quasi-species of mixed infection. In vitro results showed the replication capacity of individual clone, exclude the influence of host and other strains of quasi-species. An example from a ChiM mice study showed that monoinfection of HBV/G in ChiM mice display a very slow replication while coinfection with HBV/A remarkably enhanced the replication of HBV/G. The replication of HBV/G is heavily dependent on coinfection with other genotypes. When HBV/G superinfected on other genotypes, a rapidly takes over of HBV/G from original genotype were observed, though they are indispensable [24]. This study confirms that in a mixed infection system of different genotypes, the replication capacity of a genotype may be different from that of monoinfection. At the same time, replication capacity is not the only factor to influence which strain will become dominant. Variable recombinants found in our study may be mechanistically capable of genetic exchange, but strong selection guaranteed the elimination of hybrid genomes. The mechanism of selection in mixed infection also needs more investigation.

We found mixed HBV genotypes infection with many novel recombinants at one point in time, but just one genotype was found 18 months later. This may indicate that the detectable mixed infection and recombination has a limited time window due to the sensitivity of detection or strong selection power of the host. That's why in most studies, we can identify a major genotype in one patient. Even so, evolutionarily visible and invisible recombination of HBV could occur and play an important role by generating genetic variation or reducing mutational load. However, this study had limitation, because recombination signals were detected by RDP3 software and confirmed by split phylogenetic tree and alignment, indicating the recombinant or recombinant-like form should depend on the software. If we use another software, the results might be different.

Studies of HBV in endemic areas throughout the world have resulted in large numbers of full genome sequences available for phylogenetic analysis enabling the identification of novel, mosaic HBV genomes that appear to be the result of recombination between previously known sequences [7,25,26]. One of the most comprehensive analyses of putative HBV inter-genotype recombinants showed the existence of 24 phylogenetically independent HBV genomes involving all known human genotypes [27]. Some of these recombinants are unique to individual subjects, but some undergo expansion in specific populations and become recognized as new genotypes or subgenotypes [12,28,29,30]. Four stages in the process of generating popular HBV recombinant genomes should be recognized. The first stage is the co-circulation of different HBV strains or genotypes in the same geographic area. The second is the existence of individuals who have been infected with more than one strain of HBV. The third is the generation of a novel recombinant strain(s) within an individual. The fourth is the selection of a recombined strain with the ability to replicate and be transmitted. Our data show the natural process of the formation and selection of recombination though the recombinant strains of Y2 that appeared in 2006 that were all removed from samples in 2007.

By using phylogenetic trees and homology calculations, HBV variants infecting humans are currently classified into ten genotypes that differ from each other in nucleotide sequence by 7.5 to 13% [2,3]. There are some characteristic length differences between the genotypes that facilitates their detection and discrimination. However, as shown in Figure 2, existence of a recombinant makes the topology of the phylogenetic tree totally different from one with no recombinant. Recombinant strains obscured the definition of genotypes. Based on the algorithm creating a phylogenetic tree, sequences with high homologues cluster together. With the same logic, recombinants always clustered with the backbone parental sequence, in other words, with which they have high similarity with the larger proportion of the recombination region. Therefore, recombinants always seem to be a subgenotype of their backbone parental sequence. Similar to Y2-8 clone in Figure 2C, for recombinants with similar proportion of both parental genotypes, the sequence shows a divergent trend different from both parental genotypes.

Based on phylogenetic topology changes of different regions of HBV, it was hypothesized that some of the genotypes that are conventionally regarded as "pure," actually were recombinant. Genotype E strains show evidence of recombination with genotype D at 1950–2500. new reported genotype "I" actually belongs to genotype C. Furthermore, Subgenotype Ba possesses the recombination with genotype C at 1740 to 2485 [31,32,33]. Recombinants comprising regions with different histories have important implications for the way we think about HBV evolution. It means that there is no single phylogenetic tree that can describe the evolutionary relationships between genotypes.

In conclusion, mixed HBV genotypes infection with many novel recombinants at one point in time ended up with just one genotype 18 months later in this study. This may indicate that the detectable mixed infection and recombination have a limited time window due to the sensitivity of detection or strong selection power of the host. Also, as the recombinant or recombinant-like nature of HBV precludes the possibility of a "true" phylogenetic taxonomy, a new standard may be required for classifying HBV sequences.

Supporting Information

Figure S1 Recombination map of fragment A created by RDP software.
(TIF)

Figure S2 Recombination map of fragment B created by RDP software.
(TIF)

Figure S3 Recombination map of fragment C created by RDP software.
(TIF)

Figure S4 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S5 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S6 Split phylogenetic trees constructed by MEGA software. clone number and fragment used to construct trees are indicated beside each tree.
(TIF)

Figure S7 Alignment of fragment A(HBV nt 2813-0-1667)of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1 and light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. Y2-1'12: clones from fragment A of Y2 patients.
(DOC)

Figure S8 Alignment of fragment B(HBV nt 1822-0-257) of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1, light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. Y2-21'212: clones from fragment B of Y2 patients.
(DOC)

Figure S9 Alignment of fragment C(HBV nt 57-1818) of Y2 clones. Deep green lines are genotype C2, deep pink lines are genotype D1, light green lines are the C2 component of genotype recombinant CD1, light pink lines are the D1 component of recombinant genotype CD1. The black lines are sequence that is common to the recombining genotypes, and within which the recombination probably occurred. C2 (242): consensus sequence formed by 242 subgenotype C2 sequences from GenBank. D1 (88): consensus sequence formed by 88 subgenotype D1 sequences from GenBank. CD1 (33): consensus sequence formed by CD1 recombinant sequences from GenBank. B1B22: clones from fragment C of Y2 patients.
(DOC)

Author Contributions

Conceived and designed the experiments: ZW MM JH. Performed the experiments: BZ ZW. Analyzed the data: BZ JY JS. Contributed reagents/materials/analysis tools: HL YT. Wrote the paper: BZ YT.

References

1. Bilsel PA, Rowe JE, Fitch WM, Nichol ST (1990) Phosphoprotein and nucleocapsid protein evolution of vesicular stomatitis virus New Jersey. J Virol 64: 2498–2504.
2. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, et al. (1988) Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 69 (Pt 10): 2575–2583.
3. Norder H, Hammas B, Lofdahl S, Courouce AM, Magnius LO (1992) Comparison of the amino acid sequences of nine different serotypes of hepatitis B surface antigen and genomic classification of the corresponding hepatitis B virus strains. J Gen Virol 73 (Pt 5): 1201–1208.
4. Gerner PR, Friedt M, Oettinger R, Lausch E, Wirth S (1998) The hepatitis B virus seroconversion to anti-HBe is frequently associated with HBV genotype changes and selection of preS2-defective particles in chronically infected children. Virology 245: 163–172.
5. Liu CJ, Kao JH, Chen DS (2006) Mixed hepatitis B virus genotype infections: the more, the worse? Hepatology 44: 770.
6. Lin CL, Liu CJ, Chen PJ, Lai MY, Chen DS, et al. (2007) High prevalence of occult hepatitis B virus infection in Taiwanese intravenous drug users. J Med Virol 79: 1674–1678.
7. Wang Z, Liu Z, Zeng G, Wen S, Qi Y, et al. (2005) A new intertype recombinant between genotypes C and D of hepatitis B virus identified in China. J Gen Virol 86: 985–990.
8. Zhou B, Xiao L, Wang Z, Chang ET, Chen J, et al. (2011) Geographical and ethnic distribution of the HBV C/D recombinant on the Qinghai-Tibet Plateau. PLoS One 6: e18708.
9. Zeng GB, Wen SJ, Wang ZH, Yan L, Sun J, et al. (2004) A novel hepatitis B virus genotyping system by using restriction fragment length polymorphism patterns of S gene amplicons. World J Gastroenterol 10: 3132–3136.
10. Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, et al. (2001) A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol 82: 883–892.
11. Gunther S, Li BC, Miska S, Kruger DH, Meisel H, et al. (1995) A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. J Virol 69: 5437–5444.
12. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, et al. (2003) Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 124: 925–932.
13. Martin D, Rybicki E (2000) RDP: detection of recombination amongst aligned sequences. Bioinformatics 16: 562–563.
14. Heath L, van der Walt E, Varsani A, Martin DP (2006) Recombination patterns in aphthoviruses mirror those found in other picornaviruses. J Virol 80: 11827–11832.
15. Worobey M, Holmes EC (1999) Evolutionary aspects of recombination in RNA viruses. J Gen Virol 80 (Pt 10): 2535–2543.
16. Abdou CM, Brichler S, Mansour W, Le Gal F, Garba A, et al. (2010) A novel hepatitis B virus (HBV) subgenotype D (D8) strain, resulting from recombination between genotypes D and E, is circulating in Niger along with HBV/E strains. J Gen Virol 91: 1609–1620.
17. Phung TB, Alestig E, Nguyen TL, Hannoun C, Lindh M (2010) Genotype X/C recombinant (putative genotype I) of hepatitis B virus is rare in Hanoi, Vietnam–genotypes B4 and C1 predominate. J Med Virol 82: 1327–1333.
18. Fang ZL, Hue S, Sabin CA, Li GJ, Yang JY, et al. (2011) A complex hepatitis B virus (X/C) recombinant is common in Long An county, Guangxi and may have originated in southern China. J Gen Virol 92: 402–411.
19. Mahgoub S, Candotti D, El EM, Allain JP (2011) Hepatitis B virus (HBV) infection and recombination between HBV genotypes D and E in asymptomatic blood donors from Khartoum, Sudan. J Clin Microbiol 49: 298–306.
20. Hannoun C, Norder H, Lindh M (2000) An aberrant genotype revealed in recombinant hepatitis B virus strains from Vietnam. J Gen Virol 81: 2267–2272.
21. Banner LR, Lai MM (1991) Random nature of coronavirus RNA recombination in the absence of selection pressure. Virology 185: 441–445.
22. Kato H, Orito E, Gish RG, Sugauchi F, Suzuki S, et al. (2002) Characteristics of hepatitis B virus isolates of genotype G and their phylogenetic differences from the other six genotypes (A through F). J Virol 76: 6131–6137.

23. Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, et al. (2006) Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44: 915–924.
24. Sugiyama M, Tanaka Y, Sakamoto T, Maruyama I, Shimada T, et al. (2007) Early dynamics of hepatitis B virus in chimeric mice carrying human hepatocytes monoinfected or coinfected with genotype G. Hepatology 45: 929–937.
25. Yang J, Xing K, Deng R, Wang J, Wang X (2006) Identification of Hepatitis B virus putative intergenotype recombinants by using fragment typing. J Gen Virol 87: 2203–2215.
26. Tran TT, Trinh TN, Abe K (2008) New complex recombinant genotype of hepatitis B virus identified in Vietnam. J Virol 82: 5657–5663.
27. Simmonds P, Midgley S (2005) Recombination in the genesis and evolution of hepatitis B virus genotypes. J Virol 79: 15467–15476.
28. Morozov V, Pisareva M, Groudinin M (2000) Homologous recombination between different genotypes of hepatitis B virus. Gene 260: 55–65.
29. Owiredu WK, Kramvis A, Kew MC (2001) Hepatitis B virus DNA in serum of healthy black African adults positive for hepatitis B surface antibody alone: possible association with recombination between genotypes A and D. J Med Virol 64: 441–454.
30. Kurbanov F, Tanaka Y, Fujiwara K, Sugauchi F, Mbanya D, et al. (2005) A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. J Gen Virol 86: 2047–2056.
31. Tran TT, Trinh TN, Abe K (2008) New complex recombinant genotype of hepatitis B virus identified in Vietnam. J Virol 82: 5657–5663.
32. Tatematsu K, Tanaka Y, Kurbanov F, Sugauchi F, Mano S, et al. (2009) 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 83: 10538–10547.
33. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, et al. (2002) Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 76: 5985–5992.

Clinica Chimica Acta 413 (2012) 1796–1799

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

journal homepage: www.elsevier.com/locate/clinchim

Comparison of LecT-Hepa and FibroScan for assessment of liver fibrosis in hepatitis B virus infected patients with different ALT levels

Dongning Du [a,b,c,1], Xuejuan Zhu [a,1], Atsushi Kuno [b], Atsushi Matsuda [b], Chikayuki Tsuruno [d], Demin Yu [a], Yan Zhang [c,e], Yuzuru Ikehara [b], Yasuhito Tanaka [f], Xinxin Zhang [a,*], Hisashi Narimatsu [b,c,**]

[a] Department of Infectious Diseases, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine, 197, Ruijin Er Road, Shanghai 200025, China
[b] Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan
[c] SCSB (China) - AIST (Japan) Joint Medical Glycomics Laboratory, 800 Dong Chuan Road, Minhang, Shanghai 200240, China
[d] Product Development Div. 2, Sysmex Corporation, 4-4-4 Takatsukadai, Nishi-ku, Kobe, Hyogo 651-2271, Japan
[e] Ministry of Education Key Laboratory of Systems Biomedicine, Shanghai Center for Systems Biomedicine (SCSB), Shanghai Jiao Tong University, 800 Dong Chuan Road, Minhang, Shanghai 200240, China
[f] Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Aichi 467-8601, Japan

ARTICLE INFO

Article history:
Received 12 June 2012
Received in revised form 5 July 2012
Accepted 5 July 2012
Available online 13 July 2012

Keywords:
Liver fibrosis
Glycomarker
Hepatitis B
Cirrhosis
Alanine aminotransferase

ABSTRACT

Background: FibroScan is one of the noninvasive techniques based on the transient elastography that can assess the progression of liver fibrosis in chronic hepatitis patients in daily clinical practice. Recently, LecT-Hepa was validated as a serological glycomarker correlating well with the fibrosis stage determined by liver biopsy, and was superior to many other noninvasive biochemical markers and tests. We compared the reliability of LecT-Hepa with that of FibroScan for evaluation of liver fibrosis.
Methods: The effects of increased alanine aminotransferase (ALT) activities on LecT-Hepa and FibroScan were investigated.
Results: The areas under the receiver-operating characteristic curves, sensitivity and specificity for detecting cirrhosis, which is one of the outcomes of fibrosis estimation, were 0.82, 72.5% and 78.2% of LecT-Hepa, 0.85, 87.0% and 74.1% of FibroScan; these did not differ significantly. The count distribution of LecT-Hepa in non-cirrhosis group or cirrhosis group did not differ between the patients grouped according to their ALT levels, whereas that of FibroScan was substantially affected.
Conclusion: LecT-Hepa was confirmed as a reliable noninvasive test for the evaluation of liver fibrosis in hepatitis B virus-infected patients with comparable performance to that of FibroScan and proved to be unaffected by inflammation.

1. Introduction

It is estimated that about 2 billion people worldwide have been infected with the hepatitis B virus (HBV), and >350 million of them have chronic HBV infection [1]. In China, a seroepidemiological survey of HBV infection in 2006 showed that the prevalence of hepatitis B surface antigen positivity was 7.18%. It was estimated that 93 million people were HBV carriers, of whom 30 million were patients with chronic hepatitis B (CHB) [2]. CHB may progress to cirrhosis and hepatocellular carcinoma. An accurate method for monitoring the progression of liver fibrosis is urgently needed for the prognosis and management of chronic liver diseases. Liver biopsy is generally considered as the gold standard for assessing hepatic histology in CHB [3–5]. However, it often has limitations due to its invasiveness, risk of complications, sampling errors, and interobserver variability [6–8]. Many noninvasive methods for replacing or complementing the liver biopsy have been developed in recent years [9–12]. FibroScan (transient elastography) and FibroTest (serological marker test) have been evaluated most frequently; these methods have similar diagnostic accuracies for predicting fibrosis staging from receiver-operating characteristic (ROC) curves [13–16]. FibroTest employs a narrow and complex algorithm for 5 biochemical markers (α2-macroglobulin, apolipoprotein A1, haptoglobin, γ-glutamyl transferase, and bilirubin), which requires extensive and specialized blood analysis [17]. Recently, we developed a novel diagnostic score named LecT-Hepa for convenient and rapid monitoring of liver fibrosis progression. It is based on glyco-alteration (e.g., fucosylation and desialylation) of serum α1-acid glycoprotein

Abbreviations: HBV, hepatitis B virus; CHB, chronic hepatitis B; PLT, platelet count; AGP, α1-acid glycoprotein; LSM, liver stiffness measurement; LC, liver cirrhosis; non-LC, non-cirrhosis; DSA, *Datura stramonium* agglutinin; MAL, *Maackia amurensis* lectin; AOL, *Aspergillus oryzae* lectin.

* Corresponding author.
** Correspondence to: H. Narimatsu, Research Center for Medical Glycoscience (RCMG), National Institute of Advanced Industrial Science and Technology (AIST), 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan.
E-mail addresses: zxx10375@rjh.com.cn (X. Zhang), h.narimatsu@aist.go.jp (H. Narimatsu).
[1] These authors contributed equally to this paper.

0009-8981/$ – see front matter © 2012 Elsevier B.V. All rights reserved.
doi:10.1016/j.cca.2012.07.005

(AGP), which is assessed using a triplex lectin–antibody immunoassay [18,19]. It has been demonstrated to be well correlated with the fibrosis stage determined by liver biopsy, and verified to be more efficient by comparing with other serological methods (hyaluronic acid, tissue inhibitor of metalloproteases-1, platelet count, APRI, Forns index, Fib-4 index, and Zeng's score) in a multicenter study [20]. Here, to evaluate the reliability of LecT-Hepa for assessing liver fibrosis, we compared the diagnostic performance of LecT-Hepa and FibroScan for distinguishing cirrhosis from non-cirrhosis in a large cohort of HBV-infected Chinese patients with different serum alanine aminotransferase (ALT) levels.

2. Materials and methods

2.1. Patients

A total of 239 patients who had been positive for hepatitis B surface antigen for at least 6 months were enrolled retrospectively from Ruijin Hospital (Shanghai, China) from March 2009 to May 2011. Patients who were coinfected with another hepatitis virus or HIV, or who had excessive alcohol intake (>20 g/d), hepatocellular carcinoma, or other causes of liver diseases were excluded. For all patients, serum biochemical parameters, including the levels of aspartate aminotransferase (AST) and ALT, as well as platelet (PLT), were assessed at the time of the liver stiffness measurement. Normal values for ALT and AST ranged between 10 and 64 IU/l and between 8 and 40 IU/l, respectively, which were determined based on the manufacturer's instructions and adjusted according to the results of validation test by medical laboratory of Ruijin Hospital. Serum samples were collected at the time of the liver stiffness measurement for detection of lectins and stored at −20 °C until analysis. The patients were divided into two groups: liver cirrhosis (LC) group and non-cirrhosis (non-LC) group. The diagnosis of cirrhosis was based on clinical and morphological criteria and ultrasonography according to standard definitions [21]. The institutional ethics committees of Ruijin Hospital of Shanghai Jiao Tong University approved this study, and the informed consent was obtained from all patients.

2.2. Liver stiffness measurement

Liver stiffness was measured by transient elastography using FibroScan (EchoSens, Paris, France). The measurement depth was between 25 mm and 65 mm. For each patient, 10 validated measurements were performed. The success rate was calculated as the number of validated measurements divided by the total number of measurements. The results were expressed in kilopascals. The median value was considered representative of the elastic modulus of the liver. Only procedures with 10 validated measurements and a success rate of at least 60% were considered reliable.

2.3. Automatic acquisition of quantitative glyco-alteration of AGP

Each individual serum (5 μl) that had been stored at −20 °C was diluted 10 fold with phosphate buffered saline containing 0.2% sodium dodecyl sulfate, and then heated at 95 °C for 20 min. AGP in the resulting solution was enriched by immunoprecipitation with biotinylated anti-AGP antibody using an automated protein purification system (ED-01; GP BioSciences Ltd., Tokyo, Japan). Each elution fraction (100 μl) was kept at −80 °C until a sandwich immunoassay was performed. Subsequent to the enrichment, fibrosis-specific glyco-alteration of AGP was quantified using simultaneous lectin–antibody sandwich immunoassays for three lectins: *Datura stramonium* agglutinin (DSA), *Maackia amurensis* lectin (MAL), and *Aspergillus oryzae* lectin (AOL), by a fully automatic chemiluminescence enzyme immunoassay system (HISCL-2000i; Sysmex Co., Kobe, Japan). The criterion formula of LecT-Hepa was as before described [19]:

$$\text{LecT-Hepa} = \text{Log}_{10}[\text{AOL/DSA}] \times 8.6 - [\text{MAL/DSA}].$$

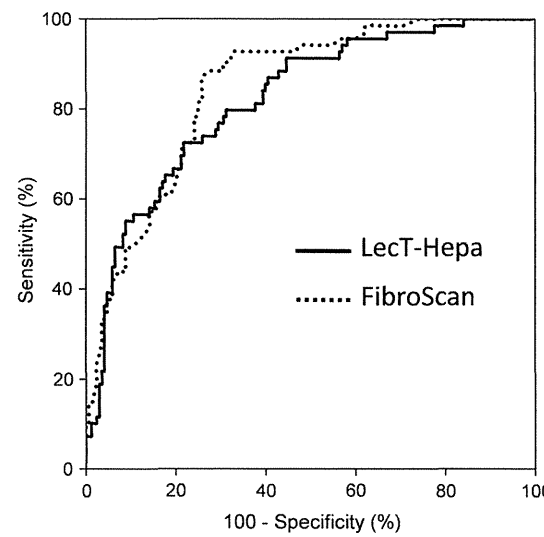
2.4. Statistical analysis

Statistical calculations were performed using software from GraphPad Prism 5 (GraphPad, San Diego, CA). A P value of <0.01 (1%) was considered to be statistically significant. The diagnostic performance of the fibrosis markers and indices were assessed using ROC curves and were then expressed as diagnostic specificity, sensitivity,

Table 1
Clinical characteristics of the patients.

Data	non-LC (n=170)	LC (n=69)	Significance non-LC vs LC
Age (y)	38.5±11.0	47.94±9.0	P<0.0001
Male sex (%)	126 (74.1%)	51 (73.9%)	-
AST (IU/l)	70.5±150.1	88.4±109.8	P=0.0002
ALT (IU/l)	111.6±213.7	88.5±116.1	P=0.1965
PLT (×10^9/l)	167.5±43.9	86.0±48.0	P<0.0001
FibroScan	10.3±8.8	27.0±19.1	P<0.0001
MAL/DSA	10.1±2.0	7.5±2.3	P<0.0001
AOL/DSA	5.1±13.5	24.0±47.6	P<0.0001

Patients were classified as non-LC or LC. LC, liver cirrhosis; non-LC, non-cirrhosis. Quantitative results are expressed as means±standard deviations or n (%).



	FibroScan	LecT-Hepa
AUC	0.85	0.82
(95% CI)	(0.797-0.897)	(0.763-0.877)
Se (%)	87.0	72.5
Sp (%)	74.1	78.2
PPV (%)	57.7	57.5
NPV (%)	93.3	87.5

Fig. 1. Receiver-operating characteristic curves of LecT-Hepa and FibroScan for distinguishing LC from non-LC. AUC, area under the receiver-operating characteristic curve; Se, sensitivity; Sp, specificity; PPV, positive predictive value; NPV, negative predictive value.

positive predictive value (PPV), negative predictive value (NPV) and area under the ROC curve (AUC) values (95% confidence interval [95% CI]).

3. Results

3.1. General characteristics

A total of 239 patients who showed evidence of chronic HBV infection and had undergone liver stiffness measurement were investigated. The mean age was 41.2 ± 11.3 y, and 177 (74%) of them were males. Among the all, 170 (71%) and 69 (29%) patients were diagnosed as non-LC and LC, respectively. Their characteristics are summarized in Table 1. Significant differences were found in Age ($P<0.0001$), AST ($P=0.0002$), PLT ($P<0.0001$), FiborScan ($P<0.0001$), MAL/DSA ($P<0.0001$), and AOL/DSA ($P<0.0001$) between the non-LC group and LC group, whereas ALT ($P=0.1965$) was not significantly different between the two groups.

3.2. Receiver-operating characteristic analysis

The overall diagnosis performances of LecT-Hepa and FibroScan were assessed using ROC curves. Fig. 1 shows the ROC curves for distinguishing LC from non-LC by both methods. The area under the ROC curve (95% CI) was 0.82 (0.763–0.877) for LecT-Hepa and 0.85 (0.797–0.897) for FibroScan. The overall diagnostic accuracies for LecT-Hepa and FibroScan were 77% and 78%, respectively. The obtained values for sensitivity, specificity, PPV, and NPV are shown in the bottom table of Fig. 1. There was no significant difference between both methods.

3.3. Effect of hepatic inflammation on the diagnostic cutoff values

Because the upper limit of the normal value for ALT level was 64 IU/l, the patients were categorized by the normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels. According to this classification, 169 patients (71%) had the normal ALT level and 70 patients (29%) had the elevated ALT level. The proportions of patients with LC in the normal and elevated ALT levels were similar (28% of normal ALT patients and 30% of elevated ALT patients). Distribution of the values obtained by each test is shown in Fig. 2. Medians of these methods increased significantly between the non-LC group and LC group (all $P<0.0001$) in the both ALT levels. LecT-Hepa values in the non-LC group ($P=0.65$) and LC group ($P=0.02$) showed no significant difference between the two ALT categories (Fig. 2A). In contrast, the FibroScan value was obviously increased with the elevation of ALT levels ($P<0.0001$) even in the same diagnostic group (Fig. 2B). Thereby, we could distinguish the LC group in the normal ALT level from non-LC group in the elevated ALT level ($P<0.0001$) by LecT-Hepa, but could not by FibroScan ($P=0.05$). Collectively, the value of FibroScan was greatly affected by the ALT levels, whereas the value for LecT-Hepa was not influenced regardless of the ALT levels.

4. Discussion

This is the first study comparing LecT-Hepa with FibroScan. These results showed the obvious advantage of LecT-Hepa in comparison with FibroScan based on robustness against fluctuation of the ALT levels with a large cohort of HBV-infected Chinese patients at different ALT levels. Thus, the diagnostic performance of LecT-Hepa was the most reliable for monitoring the progression of hepatic fibrosis.

A recent paper showed that the majority of nucleoside-naïve patients with CHB who were treated with entecavir in the long-term cohort achieved substantial histological improvement and regression of fibrosis or cirrhosis [22], suggesting that a noninvasive test for the assessment of liver fibrosis in the treated patients is required during the follow-up. The liver biopsy is limited not only by its invasive nature, but also by its accuracy. A specimen collected in a standard liver biopsy using a short, narrow-gauge needle represents a very small portion of the whole liver mass, resulting in intra- and interobserver variability and sampling errors, which account for 25% of false-negative diagnoses of cirrhosis [23–25]. Therefore, a noninvasive marker that accurately reflects the condition of the whole liver is required.

At present, FibroScan is the most intensively evaluated noninvasive method for the assessment of liver fibrosis. Its diagnostic value is considered to be superior to that of biochemical markers [26]. However, several studies noted that liver stiffness measurements using FibroScan for patients with inflammation and acute liver damage overestimate the actual stage of fibrosis and may reduce the diagnostic accuracy [27,28]. In general, a high ALT level reflects a vigorous immune response

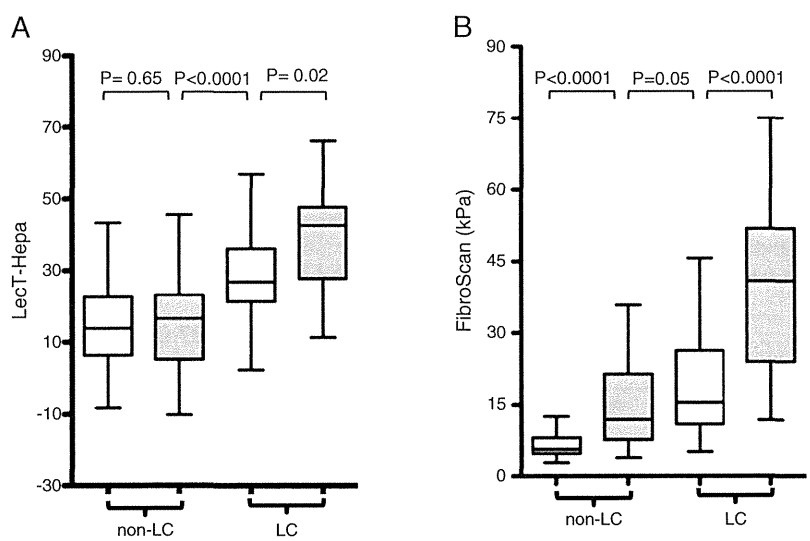


Fig. 2. Distribution of LecT-Hepa (A) and FibroScan (B) values in non-LC and LC patients with different ALT levels. The top and bottom of the whiskers are the 95th and 5th percentiles. The top and bottom of the boxes are the first and third quartiles. The size of the box represents the interquartile range within which 50% of the values are located. The line across the box indicates the median value. LC, liver cirrhosis; non-LC, non-cirrhosis. The open and gray boxes indicate normal (≤64 IU/l) and elevated (>64 IU/l) ALT levels, respectively.

to HBV and histological activity (i.e., necroinflammation). Our study obviously showed that the FibroScan values were substantially affected by ALT fluctuation. These results were also in accordance with the study of Kim et al., in which advanced fibrosis stage (F3–4) or cirrhosis showed a negative correlation with discordance between liver biopsy and FiborScan in assessing liver fibrosis in patients with CHB, and maximal activity grade 3–4 significantly influenced the liver stiffness measurement values in F3 and F4 [28]. In practice, hepatic activation and fibrosis stage should be estimated independently, as should histological diagnoses followed by a biopsy, such as the histological activity index scoring system. Thus, a marker that relies on an analysis of the specific protein content to monitor liver fibrosis should be robust against hepatic inflammation. In this context, we can explain that the reliability of LecT-Hepa is superior to that of FibroScan. LecT-Hepa has been already validated for estimating liver fibrosis using a large amount of serum specimens from patients with well-defined fibrosis stage by biopsy in a multicenter study [21]. This report led us to consider that LecT-Hepa can be a good substitute for liver biopsy. This is the reason we herein focused on the examination into the effect of hepatic inflammation on diagnosis of LC by LecT-Hepa.

In conclusion, we confirmed that LecT-Hepa is unaffected by inflammation. This suggested that LecT-Hepa is the most reliable and effective for the assessment of fibrosis progression in HBV-infected patients whose ALT levels are often fluctuated and thus can be used for routine assessments of liver fibrosis in HBV-infected patients.

Acknowledgments

The authors thank all those who helped with the measurements and the collection of serum samples, including K. Saito, S. Unno, T. Fukuda, M. Sogabe (AIST), W. Li, Y. Xu, and B. Tan (SCSB). The authors are also grateful to Y. Chiba (AIST), S. Nagai and Y. Takahama (Sysmex Co.) for critical discussion.

References

[1] Lavanchy D, Hepatitis B. virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 2004;11: 97–107.
[2] Lu FM, Zhuang H. Management of hepatitis B in China. Chin Med J-Peking 2009;122: 3-4.
[3] Afdhal N, McHutchison J, Brown R, et al. Thrombocytopenia associated with chronic liver disease. J Hepatol 2008;48:1000-7.
[4] Alberti A, Clumeck N, Collins S, et al. Short statement of the first European Consensus Conference on the treatment of chronic hepatitis B and C in HIV co-infected patients. J Hepatol 2005;42:615-24.
[5] Hoofnagle JH, Doo E, Liang TJ, Fleischer R, Lok AS. Management of hepatitis B: summary of a clinical research workshop. Hepatology 2007;45:1056-75.
[6] Colloredo G, Guido M, Sonzogni A, Leandro G. Impact of liver biopsy size on histological evaluation of chronic viral hepatitis: the smaller the sample, the milder the disease. J Hepatol 2003;39:239-44.
[7] Bravo AA, Sheth SG, Chopra S. Liver biopsy. N Engl J Med 2001;344:495-500.
[8] Piccinino F, Sagnelli E, Pasquale G, Giusti G. Complications following percutaneous liver biopsy. A multicentre retrospective study on 68,276 biopsies. J Hepatol 1986;2:165-73.
[9] Castera L. Transient elastography and other noninvasive tests to assess hepatic fibrosis in patients with viral hepatitis. J Viral Hepat 2009;16:300-14.
[10] Forns X, Ampurdanes S, Llovet JM, et al. Identification of chronic hepatitis C patients without hepatic fibrosis by a simple predictive model. Hepatology 2002;36:986-92.
[11] Patel K, Gordon SC, Jacobson I, et al. Evaluation of a panel of non-invasive serum markers to differentiate mild from moderate-to-advanced liver fibrosis in chronic hepatitis C patients. J Hepatol 2004;41:935-42.
[12] Rosenberg WM, Voelker M, Thiel R, et al. Serum markers detect the presence of liver fibrosis: a cohort study. Gastroenterology 2004;127:1704-13.
[13] Sandrin L, Fourquet B, Hasquenoph JM, et al. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. Ultrasound Med Biol 2003;29: 1705-13.
[14] Castera L, Vergniol J, Foucher J, et al. Prospective comparison of transient elastography, fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. Gastroenterology 2005;128:343-50.
[15] Friedrich-Rust M, Ong MF, Martens S, et al. Performance of transient elastography for the staging of liver fibrosis: a meta-analysis. Gastroenterology 2008;134: 960-74.
[16] Shaheen AA, Wan AF, Myers RP. FibroTest and FibroScan for the prediction of hepatitis C-related fibrosis: a systematic review of diagnostic test accuracy. Am J Gastroenterol 2007;102:2589-600.
[17] Imbert-Bismut F, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. Lancet 2001;357:1069-75.
[18] Turner GA. N-glycosylation of serum proteins in disease and its investigation using lectins. Clin Chim Acta 1992;208:149-71.
[19] Kuno A, Ikehara Y, Tanaka Y, et al. LecT-Hepa: a triplex lectin-antibody sandwich immunoassay for estimating the progression dynamics of liver fibrosis assisted by a bedside clinical chemistry analyzer and an automated pretreatment machine. Clin Chim Acta 2011;412:1767-72.
[20] Ito K, Kuno A, Ikehara Y, et al. LecT-Hepa, a glyco-marker derived from multiple lectins, as a predictor of liver fibrosis in chronic hepatitis C patients. Hepatology 2012, http://dx.doi.org/10.1002/hep.25815.
[21] Leevy CM. Diseases of the liver and biliary tract : standardization of nomenclature, diagnostic criteria, and prognosis. New York: Raven Press; 1994.
[22] Chang TT, Liaw YF, Wu SS, et al. Long-term entecavir therapy results in the reversal of fibrosis/cirrhosis and continued histological improvement in patients with chronic hepatitis B. Hepatology 2010;52:886-93.
[23] Wong VW, Vergniol J, Wong GL, et al. Diagnosis of fibrosis and cirrhosis using liver stiffness measurement in nonalcoholic fatty liver disease. Hepatology 2010;51: 454-62.
[24] Ganne-Carrie N, Ziol M, de Ledinghen V, et al. Accuracy of liver stiffness measurement for the diagnosis of cirrhosis in patients with chronic liver diseases. Hepatology 2006;44:1511-7.
[25] Bedossa P, Dargere D, Paradis V. Sampling variability of liver fibrosis in chronic hepatitis C. Hepatology 2003;38:1449-57.
[26] Colletta C, Smirne C, Fabris C, et al. Value of two noninvasive methods to detect progression of fibrosis among HCV carriers with normal aminotransferases. Hepatology 2005;42:838-45.
[27] Sagir A, Erhardt A, Schmitt M, Haussinger D. Transient elastography is unreliable for detection of cirrhosis in patients with acute liver damage. Hepatology 2008;47:592-5.
[28] Arena U, Vizzutti F, Corti G, et al. Acute viral hepatitis increases liver stiffness values measured by transient elastography. Hepatology 2008;47:380-4.

Journal of Medical Virology 84:587–595 (2012)

Multiple Intra-Familial Transmission Patterns of Hepatitis B Virus Genotype D in North-Eastern Egypt

Mostafa Ragheb,[1] Abeer Elkady,[2] Yasuhito Tanaka,[2]* Shuko Murakami,[2] Fadia M. Attia,[3] Adel A. Hassan,[1] Mohamed F. Hassan,[1] Mahmoud M. Shedid,[1] Hassan B. Abdel Reheem,[1] Anis Khan,[2] and Masashi Mizokami[4]

[1]*Department of Endemic and Infectious Disease, Suez Canal University, Ismailia, Egypt*
[2]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya, Japan*
[3]*Department of Clinical Pathology Faculty of Medicine, Suez Canal University, Ismailia, Egypt*
[4]*Research Centre for Hepatitis and Immunology, International Medical Centre of Japan Konodai Hospital, Tokyo, Japan*

The transmission rate of intra-familial hepatitis B virus (HBV) and mode of transmission were investigated in north eastern Egypt. HBV infection was investigated serologically and confirmed by molecular evolutionary analysis in family members (N = 230) of 55 chronic hepatitis B carriers (index cases). Hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) prevalence was 12.2% and 23% among family members, respectively. HBsAg carriers were prevalent in the age groups; <10 (16.2%) and 21–30 years (23.3%). The prevalence of HBsAg was significantly higher in the family members of females (19.2%) than males (8.6%) index cases ($P = 0.031$). HBsAg and anti-HBc seropositive rates were higher significantly in the offspring of females (23%, 29.8%) than those of the males index cases (4.3%, 9.8%) ($P = 0.001$, 0.003), as well as higher in the offspring of an infected mother (26.5, 31.8%) than those of an infected father (4.7%, 10.5%) ($P = 0.0006$, 0.009). No significant difference was found in HBsAg seropositive rates between vaccinated (10.6%) and unvaccinated family members (14.8%). Phylogenetic analysis of the preS2 and S regions of HBV genome showed that the HBV isolates were of subgenotype D1 in nine index cases and 14 family members. HBV familial transmission was confirmed in five of six families with three transmission patterns; maternal, paternal, and sexual. It is concluded that multiple intra-familial transmission routes of HBV genotype D were determined; including maternal, paternal and horizontal. Universal HBV vaccination should be modified by including the first dose at birth with (HBIG) administration to the newborn of mothers infected with HBV. ***J. Med. Virol. 84:587–595, 2012.*** © 2012 Wiley Periodicals, Inc.

KEY WORDS: HBV genotype D; intra-familial transmission; vaccine

INTRODUCTION

Chronic hepatitis B virus (HBV) infection is a major health problem worldwide and is affecting approximately 350 million individuals [Lee, 1997]. Infection with HBV may lead to chronic state of hepatitis in 5–10% of patients who acquired the infection in the adult life and in 80–90% of patients who acquired the infection in the infancy [Chen, 1993]. Infection with HBV can lead to a progressive liver disease including liver cirrhosis and hepatocellular carcinoma (HCC) with approximately 1 million HBV-associated deaths from HCC every year [Seeger and Mason, 2000; Kao and Chen, 2002].

Based on the proportion of the population who are seropositive for hepatitis B surface antigen (HBsAg),

Grant sponsor: The Grant for National Center For Global Health and Medicine; Grant number: 22A-9; Grant sponsor: Grant-in-Aid for Japan Society for the Promotion of Science (JSPS) Fellows; Grant number: 21.09355.

Mostafa Ragheb and Abeer Elkady contributed equally to this study.

*Correspondence to: Yasuhito Tanaka, MD, PhD, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi 1, Mizuho, Nagoya 467-8601, Japan. E-mail: ytanaka@med.nagoya-cu.ac.jp

Accepted 19 December 2011
DOI 10.1002/jmv.23234
Published online in Wiley Online Library (wileyonlinelibrary.com).

the world is divided conceptually into zones of high, intermediate, and low HBV endemic areas [Lavanchy, 2004]. In countries where the HBV infection is endemic, most infections result from the vertical transmission from the mother to the child in the peripartum period or from the infection in the early childhood. In the low HBV endemic regions, the neonatal or the childhood HBV infection is rare or even sporadic and the transmission of HBV occurs primarily among unvaccinated adults through the sexual transmission and injecting drug use [Custer et al., 2004].

Patients with chronic hepatitis B are considered to be the major reservoirs for the transmission of HBV. High incidence of infection with HBV is observed within the household contacts of chronic HBV carriers and it is not rare to have several members of the same household who have evidence of infection with HBV [Milas et al., 2000; Thakur et al., 2002]. However, the precise mechanisms of intra-familial spread have not been established clearly.

Different prophylactic strategies for controlling the HBV infection have been used by different countries depending on the prevalence of the HBV infection in each country [Poland and Jacobson, 2004]. The widespread immunization program against hepatitis B, which was implemented in more than 100 countries, was capable of dramatic reduction in the occurrence of chronic HBV infection and HCC [Zuckerman, 1997]. In Egypt, the HBV vaccine was included in 1992 in the Expanded Program of Immunization with injection at 2, 4, and 6 months of age [El Sherbini et al., 2006]. This program resulted in a significant reduction in the rate of acute symptomatic hepatitis B among the children in the age group eligible to receive the vaccine [Zakaria et al., 2007].

At least eight HBV genotypes have been identified based on the divergence of 8% or more of the entire nucleotide sequence and most of the HBV genotypes have a distinct geographical distribution [Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000]. Accumulated evidences indicated the difference in the virological characteristics among different HBV genotypes, which is reflected by the difference in the clinical outcome of infection with hepatitis B according to the infecting genotype [Miyakawa and Mizokami, 2003; Schaefer, 2005; Ozasa et al., 2006; Sugiyama et al., 2006]. However, data regarding the specificity of the transmission routes of each genotype is still scarce globally and need to be clarified.

The prevalence of HBV ranges between 2% and 6% in Egypt with the predominance of infection with HBV genotype D [Zekri et al., 2007]. It is widely known that Egypt is one of the countries with highest prevalence rate of infection with HCV in the world [el-Zayadi et al., 1992; Arthur et al., 1993; el Gohary et al., 1995]. However, the burden of HBV related progressive liver disease including liver cirrhosis and HCC in Egypt is observable either single or in a dual infection with HCV [Abdel-Wahab et al., 2000; el-Zayadi et al., 2005].

This study aimed to evaluate the prevalence of infection with HBV within the families of chronic HBV carriers in north Eastern Egypt. In addition, the intra-familial mode of transmission of HBV genotype D was also examined in the current cohort by the molecular evolutionary analyses. The impact of the HBV immunization programme in protecting this high-risk group was also investigated.

PATIENTS AND METHODS

Patients

The present study was conducted between January 2008 and June 2008 at the Communicable Disease Research and Training Centre, in Suez city. The study protocol was approved by the ethics committees of the participating institution and an informed consent was obtained from the included subjects.

Chronic HBV carriers were defined as individuals whose serum samples tested positive for HBsAg for at least 6-months period. Patients who fulfilled the criteria of chronic HBV carriers and were first detected within their families, were defined as the index cases ($n = 55$). The index cases included 40 (72.7%) men and 15 (27.3%) women. Their mean age ($\pm$ SD) was 41 ± 10.7 years and all the index cases were negative for HBeAg.

A total of 230 household contacts of the index cases were included in the study and defined as family members group. Data regarding their family relationship to the index cases, age, and the HBV vaccination history have been obtained.

According to the kinship of the family members to the index case group, the family members included 139 offspring, 4 parents, 46 spouses, 15 siblings, and 26 defined as other relatives who are living in the same house with the index cases.

Serological Methods

Serum samples were collected from the index cases and family members groups.

The Serum samples were examined for HBsAg, anti-HBc, anti-HBs, and HBeAg by the chemiluminescence enzyme immunoassay with the commercial assay kits (Fujirebio, Inc., Tokyo, Japan). The examination of the serum samples for anti-HCV and HIV was conducted using commercial kits (Abbott Laboratories, Abbott Park, IL).

Molecular Evolutionary Analysis

The HBV/DNA was extracted from 200 μl of serum samples positive for HBsAg using the QIAamp DNA MiniKit (QIGEN, Inc., Hilden, Germany), and re-suspended in 100 μl of a storage buffer (provided by the kit manufacturer).

The entire preS2 and S regions of the HBV genome (799 nucleotides; nucleotide positions 34–833) were amplified using the primers set and the conditions described previously [Sugauchi et al., 2001].

The amplified products were sequenced using Prism Big Dye (Pekrin–Elmer Applied Biosystems, Foster City, CA) in the ABI 3100 DNA automated sequencer according to the manufacturer's protocol. The sequences were aligned together with the CLUSAL X software programme [Thompson et al., 1994].

The phylogenetic tree was constructed using the neighbor joining method with Tamura-Nei's distance correction model using the Online Hepatitis Virus database (http://s2as02.genes.nig.ac.jp/) [Shin et al., 2008]. The Bootstrap values were determined on 1000 database resampling tests. The sequences of other HBV isolates used for the construction of the phylogenetic tree were retrieved from the DDBJ/EMBL/GenBank sequence database and were indicated in their accession numbers. The new nucleotide sequences data that were reported in this manuscript will appear in the DDBJ/EMBL/GenBank sequence database with accession numbers AB561825-AB561856.

Statistical Analysis

Statistical analysis was performed with the Fisher's exact probability test and the independent *t*-test for the continuous variables using the SPSS software package (SPSS, Chicago, IL). *P*-values (two-tailed) <0.05 were considered to be significant statistically.

RESULTS

The family member included 96 (41.7%) males and 134 females (58.3%). Their mean age (±SD) was 20.6 ± 14.6. The rate of seropositivity for HBsAg and anti-HBc was 12.2% (28/230) and 23% (53/230) of the family members group with no statistical significant difference between the males and females members.

Age Group Distribution of HBV Infection Within the Family Members Group

Figure 1 illustrates the HBsAg and anti-HBc prevalences among different age groups of the family members. The highest prevalence of HBsAg seropositive cases was observed in the age group, 21–30 years old; (10/43; 23.3%) followed by the age group, 0–10 years old; (11/68; 16.2%). No statistical significant difference was found in the HBsAg seropositive rates between these two age groups. The prevalence of HBsAg was 7.7% (5/65), 3.4% (1/29), and 4% (1/25) in the age groups; 11–20, 31–40, and ≥41 years old, respectively. The prevalence of anti-HBc seropositive cases was significantly increasing with the age and the highest rate was observed in the age group ≥41 years old. The prevalence of anti-HBc was 8.8% (6/68), 20% (13/65), 25.6% (11/43), 37.9% (11/29), and 48% (12/25) in the age groups; 0–10, 11–20, 21–30, 31–40, and ≥41 years old, respectively.

The HBsAg and anti-HBc seropositive rates were analyzed in the family members with respect to their relationship to the index cases (Fig. 2A). As overall, the HBsAg was positive in 6.5% (3/46) spouse of index cases, 10.8% (15/139) of the offspring, 25% (1/4) of the parents, and 40% (6/15) of the siblings (Fig. 2A).

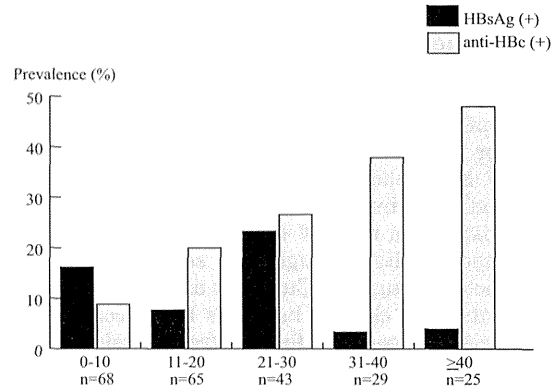


Fig. 1. Age distribution and HBV serological status among family members.

The prevalence of anti-HBc was 34.8% (16/46) in the spouse of index cases, 17.3% (24/139) in the offspring, 50% (2/4) in the parents, and 46.7% (7/15) in the siblings of the index cases (Fig. 2A).

Interestingly, the prevalence of HBsAg and anti-HBc was significantly higher in the family members of the females (19.2%, 15/78) than that of the males index cases (8.6%, 13/152; $P = 0.034$) and a trend of higher incidence of anti-HBc in the family members of the females than the males index cases (Fig. 2B). Among the offspring group, HBsAg and anti-HBc seropositive rates were significantly higher in the offspring of the females index cases (HBsAg; 23%, 11/47, anti-HBc; 29.8%, 14/47) cases than in the offspring of the males index cases (HBsAg; 4.3%, 4/92, antiHBc; 9.8%, 9/92), ($P = 0.001$, 0.003 respectively; Fig. 2C).

Further analysis was performed regarding the HBsAg seropositive rate in the offspring according to HBV infection of both one and two parents and the parent gender who is infected with HBV. Significantly higher rate of HBsAg positive (26.5%, 13/49) and anti-HBc positive (31.8%, 14/49) off spring were found in families where the mother was positive for HBsAg compared with families where the father was HBsAg positive (HBsAg; 4.7%, anti-HBc; 10.5%), ($P = 0.0006$, 0.009 respectively) (data not shown).

The seropositive rate of HBsAg was higher in the non-sexual contacts (13.6%, 25/184) of the index cases (parents, offspring, siblings, and cousins) than the sexual contacts (spouses; 6.5%, 3/46) with no statistical significant difference. Anti-HBc seropositive cases were observed more frequently in the sexual contacts (spouses) than in the non-sexual contacts (parents, offspring, siblings cousins) of the index cases. (Sexual vs. non-sexual contacts, 34.8% vs. 20.1%, $P = 0.049$) (data not shown).

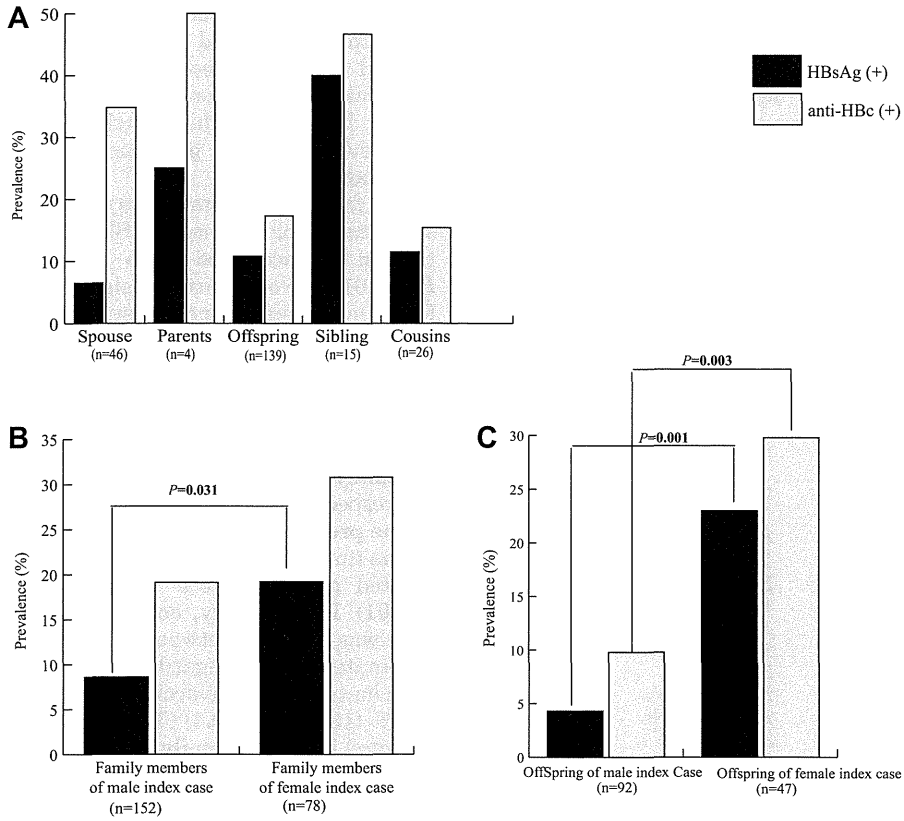


Fig. 2. Prevalence of HBsAg and anti-HBc within family members stratified by relationship to the index cases (**A**). HBV serological status of family members according to gender of the index case (**B**), and HBV serological status of the offspring according to HBV infected parent (**C**).

Molecular Evolutionary Analysis and Transmission Pattern of Hepatitis B in the Family Members Group

Eighteen index cases out of 55 (32.7%) were found to have at least one family member positive for HBsAg. The age range of these index cases was 26–56 years and 50% (9/18) of them were male (Table I). Twenty-eight family members were found to be positive for HBsAg. The data regarding the degree of relativity of each family member infected with HBV to the index case, the age of the infected family member, and the vaccination status were indicated in Table I. The mean age (±SD) of the family members with active HBV infection was 17.8 ± 13.0 years old (Table I).

The HBV genomic region of 799-nt length and spanning PreS2 and S region was amplified in 44% (8/18) of the index cases and in 50% (14/28) of the family members infected with HBV. However, the target genomic region could be amplified and sequenced simultaneously in the index cases and their related family members in six subjects. These six subjects are defined in the present report, Table I and Figure 3 as F 3, F4, F19, F35, F37, and F 43 (Table I, Fig. 3).

To confirm the family clustering, a phylogentic tree was constructed by (1) the previous mentioned sequences (2) sequences isolated from the index cases whose family members were negative for HBsAg (3) HBV nucleotide sequences isolated from HBV chronic carriers residing in different districts in Egypt (North and South) either retrieved from the data base band or further included in the present study.

The phylogenetic analysis of the preS2 and S regions of the HBV genome revealed that the HBV isolates were of subgenotype D1 (Fig. 3). Using the phylogenetic analysis, in family 4 (F4), a high homology was detected between the HBV strains isolated from the grandmother together with her daughters and her grandchildren (Fig. 3). In the Family 35 and Family 43 (F35, and F43), the father and the child harbored very closely related HBV isolates and the phylogenetic analysis suggesting that the father may have been the source of infection for his child in Family 35 (F35) and Family 43 (F43). Similarly, very closely related HBV isolates were also detected in the

TABLE I. Descriptive Analysis of the Family Members Positive for the HbsAg

Subject	Relation (gender)	Age	HBV-vaccine[a]	PreS2 + S
F3	Index (F)	42		(+)
F3-1[b]	Daughter	13	Yes	(+)
F10	Index (F)	30		(−)
F10-1	Daughter	3	Yes	(+)
F11	Index (F)	33		(+)
F11-1	Daughter	8	Yes	(−)
F11-2	Cousin	10	Yes	(−)
F30	Index (F)	42		(−)
F30-1	Son	8	Yes	(−)
F34	Index (F)	30		(−)
F34-1	Son	7	Yes	(+)
F34-2	Son	9	Yes	(+)
F48	Index (F)	30		(−)
F48-1	Son	5	Yes	(−)
F35	Index (M)	29		(+)
F35-1[b]	Daughter	5	Yes	(+)
F39	Index (M)	33		(−)
F39-1	Daughter	5	Yes	(−)
F43	Index (M)	47		(+)
F43-1[b]	Daughter	12	Yes	(+)
F55	Index (M)	56		(+)
F55-1	Daughter	12	Yes	(−)
F37	Index (M)	45		(+)
F37-1[b]	Wife	26	Yes	(+)
F36	Index (M)	31		(−)
F36-1	Brother	26	No	(−)
F36-2	Brother	28	No	(−)
F36-3	Brother	22	No	(+)
F36-4	Mother	63	No	(+)
F4	Index (F)	54		(+)
F4-1	Daughter	35	No	(+)
F4-2	Daughter	20	No	(+)
F4-3	Grandchild	6	Yes	(+)
F4-4[b]	Grandchild	4	Yes	(+)
F19	Index (M)	29		(+)
F19-1[b]	Wife	27	No	(+)
F40	Index (M)	26		(−)
F40-1	Relative	24	No	(−)
F40-2	Relative	29	No	(−)
F41	Index (F)	53		(−)
F41-1	Daughter	23	No	(−)
F41-2	Daughter	17	No	(−)
F45	Index (M)	33		(+)
F45-1	Wife	27	No	(−)
F50	Index(F)	27		(−)
F50-1	Sister	25	No	(−)

[a]HBV vaccination history is provided for the family member.
[b]Index and family members who are positive simultaneously for the PreS2and S region.

man and his wife in Families 19 and 37 (F19 and F37) (Fig. 3). The molecular evolutionary analysis of the sequences isolated from the mother and her daughter in Family 3 (F3), yielded two separate but distinct groupings of the HBV isolates, suggesting that the presence of two different HBV viral isolates infecting the mother and her daughter (Fig. 3).

Serological Markers of HBV Infection in the Vaccinated and Unvaccinated Family Members

The family members group was subdivided into two subgroups according to the history of full regimen schedule of HBV vaccination as shown in Table II; (1) A group of vaccinated family members which includes a total of 142 subjects, who received the complete HBV vaccine regimen. (2) A group of unvaccinated family members, which included 88 subjects with no previous history or incomplete regimen of HBV vaccination.

The family members in the unvaccinated group were significantly older (mean ± SD; 32.5 ± 12.5 years old) than in the vaccinated group (mean ± SD; 13.3 ± 10.4, $P = 0.012$). No statistical significant difference was found in the male gender distribution between the two groups. The anti-HBs seropositive rate was significantly higher in the vaccinated group than the unvaccinated group [69.8% (99/142) vs. 33% (29/88), respectively, $P < 0.0001$] (Table II). The mean anti-HBs titre was significantly higher in the vaccinated than unvaccinated family members (70.1 ± 129.7 vs. 21.6 ± 51.7 mIU/ml, respectively $P < 0.0001$).

The prevalence of anti-HBc was significantly higher in the unvaccinated family members compared to vaccinated groups (37.5% vs. 14.1% respectively, $P < 0.0001$). Interestingly, no statistical significant difference was detected between the vaccinated and the unvaccinated groups regarding the prevalence of HBsAg [vaccinated vs. unvaccinated; 10.6% (15/142) vs. 14.8% (13/88), $P = 0.4$] (Table II). The HBV DNA was detected in 50% of family members positive for HBsAg with no statistical significant difference between the vaccinated (53%, 8/142) and unvaccinated groups (46.2%, 6/88) (Table II).

Mutations in the "a" determinant region. The available nucleotide sequences spanning the S gene of HBV isolated from the nine vaccinated and five unvaccinated members were translated into amino acid and aligned in correspondence to the reference sequences. The amino acid substitutions in the "a" determinant region that was reported to be associated with vaccine escape mutation were not detected. However, an amino acid substitution at the second loop of "a" determinant region (T143L) was clustered in the family subject F37 (F37 and F37-1) and found in one unvaccinated family member (F4-1). Another substitution was detected in the second loop of "a" determinant region (T140I) in an unvaccinated member (F36-1). P127A substitution in first loop of the "a" determinant region was clustered in the family 43(F43 and F43-1; Fig. 4).

DISCUSSION

The investigation of the intra-familial transmission in a particular region usually reveals valuable information about the routes of HBV spread in general and may help in exploring the HBV spread problem and local peculiarities. This study is the first one in Egypt done to explore the intra-familial spread of HBV infection and inclusively HBV genotype D transmission routes in Egypt. An evaluation of the impact of the universal HBV vaccination on the intra-familial transmission of HBV was also done.

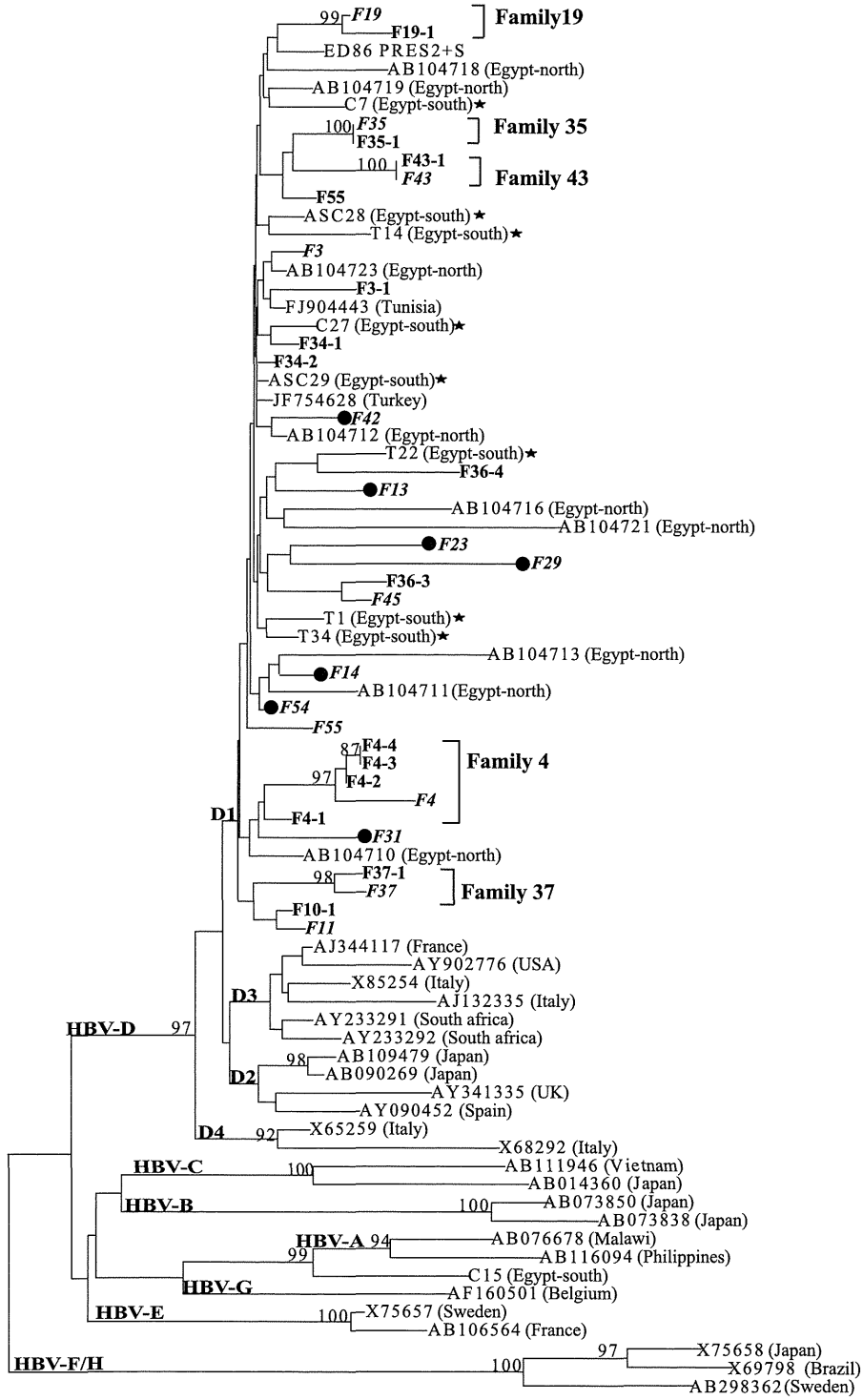


Fig. 3. Phylogenetic tree constructed by the nucleotide sequences of the partial PreS2 and S HBV genomic region. The phylogenetic tree is constructed by the neighbor joining method and significant bootstrap values (>75%) are indicated in the tree roots. HBV sequences isolated from index cases and family members are indicated in italic bold and bold fonts respectively. Reference sequences retrieved from the GenBank/EMBL/DDBJ are indicted in their accession numbers. Solid black rounds indicate sequences from index cases with family members negative for HBsAg. (★) Strains isolated from chronic hepatitis B carriers residing in Egypt south. The country origin of the reference sequences are indicated in brackets. HBV genotypes A–H are indicated in the cluster roots.

TABLE II. Comparison of Hepatitis B Serological Markers in Vaccinated Versus Unvaccinated Family Members Group

	Total (N = 230)	Vaccinated group (N = 142)	Unvaccinated group (N = 88)	*P*-value
Age[a]	20.6 ± 14.6	13.3 ± 10.4	32.5 ± 51.7	<0.0001
Gender (Male)[b]	96(41.7)	64 (45.1)	32 (36.4)	NS
Anti-HBc (+)[b]	53 (23)	20 (14.1)	33 (37.5)	<0.0001
HBsAg (+)[b]	28 (12.2)	15 (10.6)	13 (14.8)	NS
Anti-HBs (+)[b]	128 (55.7)	99 (69.8)	29(33)	<0.0001
HBV-DNA (+)[b]	14 (50)	8 (53.3)	6 (46.2)	NS

[a]Mean ± SD.
[b]N (%).

In the present study, 12.1% of the family members were infected with HBV. This incidence was much higher than that detected among the blood donors (1.4%) resident in the same area in Egypt (data not shown). Clustering of the HBV infection within the families has been described in nearby countries located within the same zone of the HBV endemicity but with different incidences; 30% in Turkey, 15.8% in Greece, and 11.9% in Iran [Alizadeh et al., 2005; Zervou et al., 2005; Ucmak et al., 2007]. An important risk factor was found to be implicated in acquiring the infection among the family was the presence of female infected with HBV. Furthermore, the higher incidence of HBsAg positive rate among the offspring of the females' index cases than that of males index cases illustrates clearly the role of the mother in the transmission of HBV. Similarly, Salkic et al. [2007] reported the same observation in his study from Bosnia [Salkic et al., 2007]. However, in Taiwan no significant difference was found in the HBsAg positivity among the offspring of the two groups, suggesting the importance of the paternal as well as the maternal transmission for the HBV intra-familial spread in Taiwan [Lin et al., 2005].

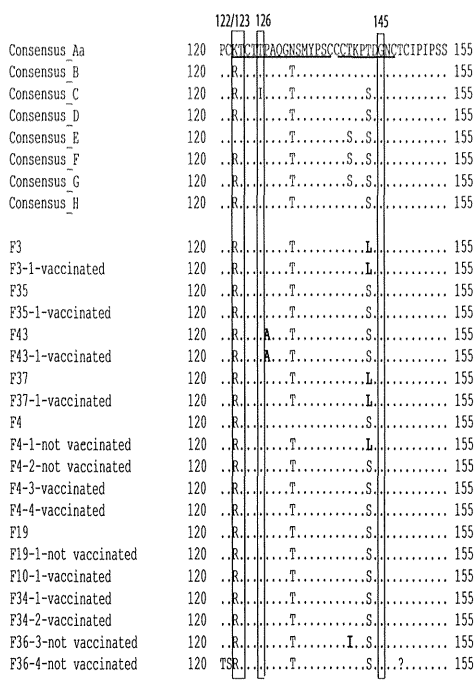


Fig. 4. The alignment of amino acid sequences of the HBV partial surface gene encompassing the "a" determinant region in the HBsAg positive family members. The upper eight sequences are consensus of the corresponding HBV genotypes Aa/A1, B, C, D, E, F, G, and H reference strain retrieved from DDBJ/GenBank database. Dots in alignment indicate identity of amino acids to the consensus sequence of genotype Aa/A1. First and second loop positions are underlined in the consensus sequence of the genotype Aa/A1 and positions of previously reported vaccine escape mutants are indicated in numbers and included in boxes.

Despite being a tedious and labor-intensive method, sequencing of the viral genomes isolated from different individuals, with the subsequent homology comparison and the phylogenetic analysis remains the golden approach for demonstrating the HBV transmission in a given population [Dumpis et al., 2001; Zampino et al., 2002; Tajiri et al., 2007].

The full length HBV sequence analysis is the gold standard for this purpose but remains a cost approach [Datta et al., 2007]. Highly variable HBV genomic region is recommended by some investigators to study the transmission event. Variability of the genomic region is affected by several factors one of which is the clinical characteristics of the studied cohort [Wu et al., 2005]. PreC/C region exhibit high variability in the cases of acute or fulminant hepatitis and thus analysis of this region is preferable for investigating the chain of recent/nosocomial fulminant cases [Bracho et al., 2006; Ozasa et al., 2006]. However, a high S gene variability is documented among the chronic hepatitis B carriers and their families, thus investigating the genotype, subgenotype, subtypes, and mutations by the sequence analysis of the S gene with further analysis by testing the constructed phylogenetic tree with the bootstrap resampling maximum-likelihood test, may provide enough confidence to prove the transmission event in the case of chronic HBV carriers [Thakur et al., 2003]. Hence, in the present study, the phylogenetic analysis of the HBV nucleotide sequences spanning the entire preS2 and S HBV genomic regions and isolated from chronic hepatitis B carriers which include index cases and their family members revealed the infection with HBV genotype D which coincides with the previous

data regarding the predominance of infection with HBV genotype D in Egypt [Saudy et al., 2003]. In addition, the phylogenetic analysis documented the presence of three different patterns of HBV genotype D transmission within the families in Egypt; maternal transmission (from mother to child as in the family 4), paternal transmission (from father to child as in family 35 and family 43) and spousal transmission (between spouses as in family 19 and family 37). This was different from the transmission pattern characteristics of genotype D in Uzbekistan where the horizontal transmission was the predominant route of infection with HBV genotype D within a family [Avazova et al., 2008].

The Data regarding the difference of transmission routes of HBV infection between different genotypes are controversial and scarce. Based on the findings that the patients infected with HBV genotype C may exhibit delayed HBeAg seroconversion decades later than the patients infected with other genotypes, Livingston et al. [2007] speculated that genotype C is the most responsible for the perinatal transmission and that the other genotypes (A, B, D, and F) are mainly transmitted horizontally [Livingston et al., 2007]. A recent study has shown a different data through exploring that both genotypes B and C can be transmitted by maternal and horizontal routes [Wen et al., 2011]. Whether different HBV genotypes have different transmission routes remains a question, which needs further global studies to clarify this interesting and important issue.

In an attempt to evaluate the influence of the universal vaccination on the intra-familial HBV infection, it was surprising to find a high prevalence rate of HBsAg among the vaccinated members with no significant difference when compared to the unvaccinated group. In an agreement with the present data, El Sherbini et al. [2006] reported the unchangeable prevalence of HBsAg among the vaccinated school children across a decade despite the significant decrease of the anti-HBc rate [El Sherbini et al., 2006]. The possible explanation for this vaccine failure is the acquiring of the HBV infection in the lag period between the birth and the time of receiving the first HBV vaccine dose at the age of 2 months. Supporting our explanation is the recent data coming from Taiwan where a different HBV infection prophylactic strategy is applied by administrating the first dose of the HBV vaccine at birth with the administration of the hepatitis B immunoglobulin to the infants born to the HBeAg positive mother within 24 hr after birth. The recent study has clearly demonstrated that the current HBV prophylactic strategy in Taiwan was capable of reducing the intra-familial HBV transmission and reducing the overall HBsAg positive rate among the infants [Mu et al., 2011]. In Japan, the extension of the active and passive immunization to the babies born to HBeAg negative mother had greatly reduced the HBsAg prevalence to 0.2% of blood donors younger than 19 years old [Noto et al., 2003; Matsuura et al., 2009]. The present study recommends the changing of the current HBV prophylactic policy in Egypt. It would be needed to provide the first dose of the HBV vaccine at birth together with screening for HBV infection markers prenatally and administration of the HBIG to the infants born from HBeAg-positive mothers. The documented role of the HBV spousal transmission in the present study by the phylogenetic analysis (Family 19 and Family 37), coincides with the recent data conducted in Egypt that the first sexual contact with an infected spouse was a significant risk factor for infection with HBV among females and may further emphasize the importance of the premarital screening for HBV in Egypt [Paez Jimenez et al., 2009]. Investigating the " a" determinant region of viral isolates retrieved from the vaccinated members infected with HBV provides no evidence of breakthrough infection by previously reported vaccine escape mutant virus [Carman et al., 1990].

In conclusion, the present study has clearly explored the role of the HBV intra-familial transmission and spread in north Eastern Egypt. Three patterns of HBV transmission were determined in the current cohort infected with HBV genotype D; maternal, paternal, and spousal. The present study recommends the change of the current prophylactic policy against the HBV infection in Egypt by including the first dose of HBV vaccine at birth, screening of pregnant women for HBsAg and the administration of HBIG to the infants born from HBeAg positive mothers within 24 hr after birth. Further studies are needed globally to determine the transmission patterns of different HBV genotypes and locally in different districts in Egypt to explore the impact of familial transmission in HBV infection in Egypt.

REFERENCES

Abdel-Wahab M, el-Enein AA, Abou-Zeid M, el-Fiky A, Abdallah T, Fawzy M, Fouad A, Sultan A, Fathy O, el-Ebidy G, elghawalby N, Ezzat F. 2000. Hepatocellular carcinoma in Mansoura-Egypt: Experience of 385 patients at a single center. Hepatogastroenterology 47:663–668.

Alizadeh AH, Ranjbar M, Ansari S, Alavian SM, Shalmani HM, Hekmat L, Zali MR. 2005. Intra-familial prevalence of hepatitis B virologic markers in HBsAg positive family members in Nahavand, Iran World J Gastroenterol 11:4857–4860.

Arthur RR, el-Sharkawy MS, Cope SE, Botros BA, Oun S, Morrill JC, Shope RE, Hibbs RG, Darwish MA, Imam IZ. 1993. Recurrence of Rift Valley fever in Egypt. Lancet 342:1149–1150.

Avazova D, Kurbanov F, Tanaka Y, Sugiyama M, Radchenko I, Ruziev D, Musabaev E, Mizokami M. 2008. Hepatitis B virus transmission pattern and vaccination efficiency in Uzbekistan. J Med Virol 80:217–224.

Bracho MA, Gosalbes MJ, Gonzalez F, Moya A, Gonzalez-Candelas F. 2006. Molecular epidemiology and evolution in an outbreak of fulminant hepatitis B virus. J Clin Microbiol 44:1288–1294.

Carman WF, Zanetti AR, Karayiannis P, Waters J, Manzillo G, Tanzi E, Zuckerman AJ, Thomas HC. 1990. Vaccine-induced escape mutant of hepatitis B virus. Lancet 336:325–329.

Chen DS. 1993. From hepatitis to hepatoma: Lessons from type B viral hepatitis. Science 262:369–370.

Custer B, Sullivan SD, Hazlet TK, Iloeje U, Veenstra DL, Kowdley KV. 2004. Global epidemiology of hepatitis B virus. J Clin Gastroenterol 38:S158–S168.

Datta S, Banerjee A, Chandra PK, Chakravarty R. 2007. Selecting a genetic region for molecular analysis of hepatitis B virus transmission. J Clin Microbiol 45:687; author reply 688.

Dumpis U, Holmes EC, Mendy M, Hill A, Thursz M, Hall A, Whittle H, Karayiannis P. 2001. Transmission of hepatitis B virus infection in Gambian families revealed by phylogenetic analysis. J Hepatol 35:99–104.

el Gohary A, Hassan A, Nooman Z, Lavanchy D, Mayerat X, el Ayat A, Fawaz N, Gobran F, Ahmed M, Kawano F, Ragheb M, Elkady A, Tanaka Y, Murakami S, Attia FM, Hassan AA, Hassan MF, Shedid MM, Abdel Reheem HB, Khan A, Mizokami M. 1995. High prevalence of hepatitis C virus among urban and rural population groups in Egypt. Acta Trop 59:155–161.

El Sherbini A, Mohsen SA, Seleem Z, Ghany AA, Moneib A, Abaza AH. 2006. Hepatitis B virus among schoolchildren in an endemic area in Egypt over a decade: Impact of hepatitis B vaccine. Am J Infect Control 34:600–602.

el-Zayadi A, Selim O, Rafik M, el-Haddad S. 1992. Prevalence of hepatitis C virus among non-A, non-B-related chronic liver disease in Egypt. J Hepatol 14:416–417.

el-Zayadi AR, Badran HM, Barakat EM, Attia Mel D, Shawky S, Mohamed MK, Selim O, Saeid A. 2005. Hepatocellular carcinoma in Egypt: A single center study over a decade. World J Gastroenterol 11:5193–5198.

Kao JH, Chen DS. 2002. Global control of hepatitis B virus infection. Lancet Infect Dis 2:395–403.

Lavanchy D. 2004. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J Viral Hepat 11:97–107.

Lee WM. 1997. Hepatitis B virus infection. N Engl J Med 337:1733–1745.

Lin CL, Kao JH, Chen BF, Chen PJ, Lai MY, Chen DS. 2005. Application of hepatitis B virus genotyping and phylogenetic analysis in intrafamilial transmission of hepatitis B virus. Clin Infect Dis 41:1576–1581.

Livingston SE, Simonetti JP, Bulkow LR, Homan CE, Snowball MM, Cagle HH, Negus SE, McMahon BJ. 2007. Clearance of hepatitis B e antigen in patients with chronic hepatitis B and genotypes A, B, C, D, and F. Gastroenterology 133:1452–1457.

Matsuura K, Tanaka Y, Hige S, Yamada G, Murawaki Y, Komatsu M, Kuramitsu T, Kawata S, Tanaka E, Izumi N, Okuse C, Kakumu S, Okanoue T, Hino K, Hiasa Y, Sata M, Maeshiro T, Sugauchi F, Nojiri S, Joh T, Miyakawa Y, Mizokami M. 2009. Distribution of hepatitis B virus genotypes among patients with chronic infection in Japan shifting toward an increase of genotype A. J Clin Microbiol 47:1476–1483.

Milas J, Ropac D, Mulic R, Milas V, Valek I, Zoric I, Kozul K. 2000. Hepatitis B in the family. Eur J Epidemiol 16:203–208.

Miyakawa Y, Mizokami M. 2003. Classifying hepatitis B virus genotypes. Intervirology 46:329–338.

Mu SC, Wang GM, Jow GM, Chen BF. 2011. Impact of universal vaccination on intrafamilial transmission of hepatitis B virus. J Med Virol 83:783–790.

Norder H, Courouce AM, Magnius LO. 1994. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 198:489–503.

Noto H, Terao T, Ryou S, Hirose Y, Yoshida T, Ookubo H, Mito H, Yoshizawa H. 2003. Combined passive and active immunoprophylaxis for preventing perinatal transmission of the hepatitis B virus carrier state in Shizuoka, Japan during 1980–1994. J Gastroenterol Hepatol 18:943–949.

Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, Mayumi M. 1988. Typing hepatitis B virus by homology in nucleotide sequence: Comparison of surface antigen subtypes. J Gen Virol 69:2575–2583.

Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang JH, Hige S, Kuramitsu T, Suzuki K, Tanaka E, Okada S, Tokita H, Asahina Y, Inoue K, Kakumu S, Okanoue T, Murawaki Y, Hino K, Onji M, Yatsuhashi H, Sakugawa H, Miyakawa Y, Ueda R, Mizokami M. 2006. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. Hepatology 44:326–334.

Paez Jimenez A, El-Din NS, El-Hoseiny M, El-Daly M, Abdel-Hamid M, El Aidi S, Sultan Y, El-Sayed N, Mohamed MK, Fontanet A. 2009. Community transmission of hepatitis B virus in Egypt: Results from a case–control study in Greater Cairo. Int J Epidemiol 38:757–765.

Poland GA, Jacobson RM. 2004. Clinical practice: Prevention of hepatitis B with the hepatitis B vaccine. N Engl J Med 351:2832–2838.

Salkic NN, Zildzic M, Muminhodzic K, Pavlovic-Calic N, Zerem E, Ahmetagic S, Mott-Divkovic S, Alibegovic E. 2007. Intrafamilial transmission of hepatitis B in Tuzla region of Bosnia and Herzegovina. Eur J Gastroenterol Hepatol 19:113–118.

Saudy N, Sugauchi F, Tanaka Y, Suzuki S, Aal AA, Zaid MA, Agha S, Mizokami M. 2003. Genotypes and phylogenetic characterization of hepatitis B and delta viruses in Egypt. J Med Virol 70: 529–536.

Schaefer S. 2005. Hepatitis B virus: Significance of genotypes. J Viral Hepat 12:111–124.

Seeger C, Mason WS. 2000. Hepatitis B virus biology. Microbiol Mol Biol Rev 64:51–68.

Shin IT, Tanaka Y, Tateno Y, Mizokami M. 2008. Development and public release of a comprehensive hepatitis virus database. Hepatol Res 38:234–243.

Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. 2000. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J Gen Virol 81: 67–74.

Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S, Kimura Y, Ueda R, Butterworth LA, Cooksley WG. 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: Complete genome sequence and phylogenetic relatedness. J Gen Virol 82:883–892.

Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK, Gish RG, Kramvis A, Shimada T, Izumi N, Kaito M, Miyakawa Y, Mizokami M. 2006. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44:915–924.

Tajiri H, Tanaka Y, Kagimoto S, Murakami J, Tokuhara D, Mizokami M. 2007. Molecular evidence of father-to-child transmission of hepatitis B virus. J Med Virol 79:922–926.

Thakur V, Guptan RC, Malhotra V, Basir SF, Sarin SK. 2002. Prevalence of hepatitis B infection within family contacts of chronic liver disease patients – Does HBeAg positivity really matter? J Assoc Physicians India 50:1386–1394.

Thakur V, Kazim SN, Guptan RC, Malhotra V, Sarin SK. 2003. Molecular epidemiology and transmission of hepatitis B virus in close family contacts of HBV-related chronic liver disease patients. J Med Virol 70:520–528.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Ucmak H, Faruk Kokoglu O, Celik M, Ergun UG. 2007. Intra-familial spread of hepatitis B virus infection in eastern Turkey. Epidemiol Infect 135:1338–1343.

Wen WH, Chen HL, Ni YH, Hsu HY, Kao JH, Hu FC, Chang MH. 2011. Secular trend of the viral genotype distribution in children with chronic hepatitis B virus infection after universal infant immunization. Hepatology 53:429–436.

Wu W, Chen Y, Ruan B, Li LJ. 2005. Gene heterogeneity of hepatitis B virus isolates from patients with severe hepatitis B. Hepatobiliary Pancreat Dis Int 4:530–534.

Zakaria S, Fouad R, Shaker O, Zaki S, Hashem A, El-Kamary SS, Esmat G, Zakaria S. 2007. Changing patterns of acute viral hepatitis at a major urban referral center in Egypt. Clin Infect Dis 44:e30–e36.

Zampino R, Lobello S, Chiaramonte M, Venturi-Pasini C, Dumpis U, Thursz M, Karayiannis P. 2002. Intra-familial transmission of hepatitis B virus in Italy: Phylogenetic sequence analysis and amino-acid variation of the core gene. J Hepatol 36:248–253.

Zekri AR, Hafez MM, Mohamed NI, Hassan ZK, El-Sayed MH, Khaled MM, Mansour T. 2007. Hepatitis B virus (HBV) genotypes in Egyptian pediatric cancer patients with acute and chronic active HBV infection. Virol J 4:74.

Zervou EK, Gatselis NK, Xanthi E, Ziciadis K, Georgiadou SP, Dalekos GN. 2005. Intrafamilial spread of hepatitis B virus infection in Greece. Eur J Gastroenterol Hepatol 17:911–915.

Zuckerman AJ. 1997. Prevention of primary liver cancer by immunization. N Engl J Med 336:1906–1907.

PLOS ONE

Genetic Association of Human Leukocyte Antigens with Chronicity or Resolution of Hepatitis B Infection in Thai Population

Nawarat Posuwan[1,3,4], Sunchai Payungporn[2,3], Pisit Tangkijvanich[2,3], Shintaro Ogawa[1], Shuko Murakami[1], Sayuki Iijima[1], Kentaro Matsuura[1], Noboru Shinkai[1], Tsunamasa Watanabe[1], Yong Poovorawan[4], Yasuhito Tanaka[1]*

1 Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan, **2** Research Unit of Hepatitis and Liver Cancer, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, **3** Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, **4** Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand

Abstract

Background: Previous studies showed that single nucleotide polymorphisms (SNPs) in the *HLA-DP*, *TCF19* and *EHMT2* genes may affect the chronic hepatitis B (CHB). To predict the degree of risk for chronicity of HBV, this study determined associations with these SNPs.

Methods: The participants for this study were defined into 4 groups; HCC (n = 230), CHB (n = 219), resolved HBV infection (n = 113) and HBV uninfected subjects (n = 123). The *HLA-DP* SNPs (rs3077, rs9277378 and rs3128917), *TCF19* SNP (rs1419881) and *EHMT2* SNP (rs652888) were genotyped.

Results: Due to similar distribution of genotype frequencies in HCC and CHB, we combined these two groups (HBV carriers). The genotype distribution in HBV carriers relative to those who resolved HBV showed that rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, $p<0.001$ and OR = 0.47, $p<0.001$). The other SNPs rs3128917, rs1419881 and rs652888 were not associated with HBV carriers.

Conclusions: Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population.

Citation: Posuwan N, Payungporn S, Tangkijvanich P, Ogawa S, Murakami S, et al. (2014) Genetic Association of Human Leukocyte Antigens with Chronicity or Resolution of Hepatitis B Infection in Thai Population. PLoS ONE 9(1): e86007. doi:10.1371/journal.pone.0086007

Editor: Man-Fung Yuen, The University of Hong Kong, Hong Kong

Received September 9, 2013; **Accepted** December 4, 2013; **Published** January 23, 2014

Funding: This work was supported by grants from The JSPS RONPAKU (Dissertation PhD) Program; the Ratchadapiseksompotch Endowment Fund of Chulalongkorn University [RES560530155-AM and RES560530093-HR); Higher Education Research Promotion and National Research University Project of Thailand Office of the Higher Education Commission [HR1155A-55 and HR1162A-55]; Thailand Research Fund [DPG5480002 and BRG5580005]; Chulalongkorn University, Integrated Innovation Academic Center, Chulalongkorn University Centenary Academic Development Project [CU56-HR01]; King Chulalongkorn Hospital and the Office of the National Research Council of Thailand (NRCT); grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan and the Ministry of Education, Culture, Sports, Science and Technology, Japan. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: ytanaka@med.nagoya-cu.ac.jp

Introduction

The hepatitis B virus (HBV) is one of the most common causes of chronic hepatitis B (CHB), liver cirrhosis and hepatocellular carcinoma (HCC). Globally more than 2 billion people have been infected with HBV and 378 million are suffering from chronic hepatitis. Over 600,000 people die each year because of HBV infection. In high prevalence areas such as the central Asian republics, Southeast Asia, Sub-Saharan Africa and the Amazon basin over 8% of the population may be HBV carriers [1]. The main route of HBV infection is vertical transmission from mother to infant and horizontal transmission between children, whereby 90% will develop chronic hepatitis as infants or in early childhood and never clear the virus [1–3]. In contrast, 15% of HBV infections in adulthood develop into chronic hepatitis with viral persistence.

The frequency of HBV infection which develops into chronic hepatitis depends on the age at which the person is infected [1,2]. However, the factors determining HBV persistence or clearance are not clearly understood [4–6]. Risk factors for viral persistence include the following: virological factors (viral load, genotype, viral gene mutations and co-infection with another virus), host factors (age at infection, gender, immune status and genetic variability) and extrinsic factors (e.g. alcohol consumption and chemotherapy) [7]. Whether viral infection results in acute or chronic infection also depends on cellular immune responses influenced by human leukocyte antigen (*HLA*) class I and II molecules which must present the viral antigens to CD8+ T cells and CD4+ T cells, respectively [8]. The genes encoding *HLA* are the most

polymorphic in the human genome, presumably in order to be able to respond to all potential foreign antigens [9].

Recently, many genome-wide association studies (GWAS) have been performed to seek associations between human genetic variation and the outcome of HBV infection [10–15]. Studies in the Japanese population showed that 11 single nucleotide polymorphisms (SNPs) located within or around the *HLA-DPA1* and *HLA-DPB1* loci are significantly associated with the occurrence of CHB. Of these 11 SNPs, the most strongly associated with the outcome of HBV infection were rs9277535 and rs3128917 in *HLA-DPB1* and rs3077 in *HLA-DPA1* [10].

Thereafter, GWAS studies in the Korean population confirmed the presence of these host factors related to HBV outcome and reported two new SNPs significantly associated with CHB within the *HLA* region, namely rs1419881 and rs652888 in transcription factor 19 (*TCF19*) and euchromatic histone-lysine methyltransferase 2 (*EHMT2*), respectively [16]. *TCF19* (or transcription factor SC1) is a *trans*-activating factor that mainly influences the transcription of genes required for late growth regulation at the G1-S checkpoint and during S phase [17]. *EHMT2* is a histone methyltransferase responsible for mono- and di-methylation of H3K9 (lysine at 9th residue of histone subunit 3) in euchromatin [18], which modifies the conformation of chromatin from its tightly packed form, heterochromatin, and thus influences gene repression or transcriptional silencing [19].

In the present study, we determined associations between the SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), *TCF19* (rs1419881) and *EHMT2* (rs652888) in HBV infected patients compared to those with resolved infections and those who had never been infected.

Materials and Methods

Ethics Statement

This study was approved by the Institutional Review Board of the Faculty of Medicine, University (Bangkok, Thailand) code IRB.455/54. Written informed consent was obtained from each patient and all samples were anonymized.

Sample Collection

All blood samples were negative for hepatitis C virus and human immunodeficiency virus. Subjects were defined into 4 groups: 230 hepatitis B surface antigen (HBsAg)-positive HCC, and 219 CHB who had been HBsAg-positive for at least 6 months were recruited at the King Chulalongkorn Memorial Hospital, whereas patients with resolved HBV and uninfected subjects were from the Thai Red Cross Society and from the north-eastern part of Thailand (age>40 years) which had been screened by Immunoassay (Architect i2000SR, Abbott, USA.) for HBsAg, antibody to hepatitis B surface antigen (anti-HBs) and antibody to hepatitis B core protein (anti-HBc). Of these subjects, 113 were negative for HBsAg but positive for anti-HBc and/or positive for anti-HBs after resolution of infection, while 123 uninfected subjects were all negative for HBsAg, anti-HBc and anti-HBs. All samples in this study were collected from subjects who have lived at the same area in Thailand, suggesting that the genetic background would be balanced between a case and control.

Genotyping assays

DNA was extracted from peripheral blood mononuclear cell using phenol-chloroform DNA extraction. The concentration of DNA was determined by NanoDrop 2000c spectrophotometer (Thermo Scientific, Wilmington, DE). We determined SNPs of *HLA-DPA1* (rs3077), *HLA-DPB1* (rs9277378 and rs3128917), and the genes *TCF19* (rs1419881) and *EHMT2* (rs652888) by commercial TaqMan PCR assays (Applied Biosystems, USA). In this study we investigated *HLA-DPB1* (rs9277378) because this SNP had a high level of linkage disequilibrium with rs9277535 ($D' = 1.00$, $R^2 = 0.954$) [20] and was clearly detectable by the TaqMan assay rather than rs9277535.

Statistical analyses

In this study, Hardy-Weinberg equilibrium was performed on each SNP. The Chi-square test of independence and Odds Ratio (OR) from two-by-two tables for comparisons between case and control groups was performed using Microsoft Excel. Statistical significance was defined by $P<0.05$. The calculated of possibility level was established using Chi-square contingency table analysis.

Results

Subjects were defined into 4 groups: group 1) HCC (age = 58.2±12 years, 190/230 (82.6%) male); group 2) CHB (age = 46.6±10 years, 144/219 (65.7%) male); group 3) those with resolved HBV (age = 48.2±6 years, 83/113 (73.5%) male); and group 4) HBV uninfected subjects (age = 46.7±6 years, 73/123 (59.3%) male). The details are given in Table 1. To find the genetic factor associated with chronicity of HBV infection, however, the two groups (group 1 and 2) were combined (designated "HBV carriers"). Indeed, according to the frequencies of minor alleles of the SNPs in the *HLA-DP*, *TCF19* and *EHMT2* genes listed in Table 2, the frequencies of minor alleles of these 5 SNPs in HCC and CHB were similar (data shown in Table S1). The composite HBV carriers group had a minor allele frequency for rs3077 and rs9277378 lower than in groups 3 and 4 (OR = 0.57, 95% CI = 0.42–0.78, $p<0.001$ and OR = 0.63, 95% CI = 0.47–0.85, $p = 0.008$ for rs3077, OR = 0.59, 95% CI = 0.44–0.81, $p = 0.001$ and OR = 0.56, 95% CI = 0.42–0.75, $p<0.001$ for rs9277378, respectively). In contrast, the minor allele frequency for rs1419881 in HBV carriers was similar to group 3 (OR = 0.80, 95% CI = 0.60–1.08, $p = 0.142$) but lower than in group 4 (OR = 0.64, 95% CI = 0.48–0.85, $p = 0.002$). Moreover, minor allele frequency for rs3128917 and rs652888 in HBV carriers was comparable to groups 3 and 4 (OR = 1.14, 95% CI = 0.85–1.53, $p = 0.371$ and OR = 1.06, 95% CI = 0.80–1.41, $p = 0.673$ for rs3128917; OR = 1.14, 95% CI = 0.84–1.55, $p = 0.400$ and OR = 1.12, 95% CI = 0.83–1.50, $p = 0.471$ for rs652888, respectively).

The results of Hardy-Weinberg equilibrium analysis of each SNPs were shown in Table 3. All data were over 0.01 ($p>0.01$), indicating that the frequencies did not deviate from Hardy-Weinberg equilibrium. The genotype distribution in HBV carriers compared to subjects with HBV resolution showed that both rs3077 and rs9277378 were significantly associated with protective effects against CHB in minor dominant model (OR = 0.45, 95% CI = 0.30–0.69, $p<0.001$ for rs3077 and OR = 0.47, 95% CI = 0.31–0.72, $p<0.001$ for rs9277378, are described in Table 3), suggesting that major homozygous genotypes were risk factors with the chronicity of HBV. The other SNPs rs3128917, rs1419881 and rs652888 were not associated against HBV carrier status (OR = 1.22, 95% CI = 0.76–1.97, $p = 0.413$ for rs3128917, OR = 0.67, 95% CI = 0.42–1.06, $p = 0.084$ for rs1419881 and OR = 1.31, 95% CI = 0.87–2.00, $p = 0.198$ for rs652888, respectively).

The genotype frequencies for 5 SNPs are shown in Table 3. Comparing HBV carriers with uninfected subjects showed that rs3077, rs9277378 and rs1419881 were all protectively associated with chronic HBV infection (OR = 0.63, 95% CI = 0.42–0.95,

Table 1. Characteristics of participants in HCC, CHB, resolved HBV and HBV uninfected subjects in Thailand.

	HCC (n = 230)	CHB[a] (n = 219)	Resolved[b] (n = 113)	Uninfected[c] (n = 123)
Age (years)	58.2±12	46.6±10	48.2±6	46.7±6
Male	190 (82.6%)	144 (65.7%)	83 (73.5%)	73 (59.3%)
HBsAg positive	230 (100%)	219 (100%)	0	0
ALT>40 (IU/L)	43 (18.7%)	61 (27.8%)	-	-
Alb (g/dl)	3.7 (2.5–5.6)	4.5 (3–5.2)	-	-
TB (mg/dl)	1.2 (0.17–14.8)	0.56 (0.2–2.67)	-	-

Abbreviation: HCC, hepatocellular carcinoma; CHB, chronic hepatitis B; HBsAg, hepatitis B surface antigen; ALT, Alanine transaminase; Alb, Albumin; TB, Total bilirubin.
[a]Defined as chronic hepatitis B includes chronic HBV infection but not cirrhosis and HCC.
[b]Defined as HBsAg negative but anti-HBc or/and anti-HBs positive.
[c]Defined as any HBV serological markers negative.
doi:10.1371/journal.pone.0086007.t001

$p = 0.025$ for rs3077 and OR = 0.55, 95% CI = 0.36–0.82, $p = 0.003$ for rs9277378 and OR = 0.57, 95% CI = 0.36–0.90, $p = 0.015$ for rs1419881, respectively). Comparing HBV carriers and uninfected subjects rather than those with resolved infection regarding rs1419881 was significantly protective association against CHB, but rs3128917 and rs652888 were not associated against CHB (OR = 1.58, 95% CI = 1.02–2.46, $p = 0.042$ for rs3128917 and OR = 1.09, 95% CI = 0.65–1.82, $p = 0.080$ for rs652888). When we consider the Bonferroni corrections (5 SNPs), however, the P value for rs1419881 did not reach the level of significant difference (0.015>0.05/5) between HBV carriers and HBV uninfected subjects. These data suggested that other SNPs, rs1419881, rs3128917 and rs652888 were not associated with HBV carriers in this study.

Results of meta-analysis for 3 SNPs (rs3077, rs9277378 and rs3128917) in the *HLA* gene were shown in Table S2 and S3; HBV carriers were compared to HBV resolved or HBV uninfected subjects, respectively. While the other 2 SNPs were published only from Korean population, thus the meta-analysis appeared only between HBV carriers and HBV uninfected subjects. All SNPs analyzed by the meta-analysis were significantly associated with HBV carriers.

The associations between these 5 SNPs and HBV status are depicted graphically in Figure S1. Each histogram compares HBV carriers with subjects that have resolved HBV infection or were never infected. The results showed that the minor dominant model of rs3077 and rs9277378 was highly protective associated against chronic HBV, while no significant associations were observed with rs3128917 and rs652888. Furthermore, comparing the frequency of rs1419881 between HBV carriers and uninfected subjects also revealed its association against chronic HBV infection but the association with resolved HBV did not achieve statistical significance.

Discussion

Genetic variations of rs3077 and rs9277378, but not rs3128917, rs1419881 and rs652888, were significantly associated with HBV carriers relative to resolved HBV in Thai population. In the human genome, single nucleotide polymorphisms are found in every 300–570 nucleotides. Many SNPs have no effect on the function of the encoded proteins, but some variants do appear in regulatory or coding part of the gene and affect gene expression level or protein function which can give rise to disease [21] such as the 3 SNPs including rs3077, rs9277378 and rs3128917 in *HLA-*

Table 2. Minor allele frequencies in HBV carriers, resolved HBV and uninfected subjects in Thailand.

						HBV carriers vs. Resolved		HBV carriers vs. Uninfected	
SNPs	Gene	Minor alleles[a]	HBV carriers[b] (2n = 898)	Resolved (2n = 226)	Uninfected (2n = 246)	OR (95% CI)	*P* values	OR (95% CI)	*P* values
rs3077	*HLA-DPA1*	T	227 (25.3%)	84 (37.2%)	86 (35.0%)	0.57 (0.42–0.78)	<0.001	0.63 (0.47–0.85)	0.008
rs9277378	*HLA-DPB1*	A	237 (26.4%)	85 (37.6%)	96 (39.0%)	0.59 (0.44–0.81)	0.001	0.56 (0.42–0.75)	<0.001
rs3128917	*HLA-DPB1*	G	459 (51.1%)	108 (47.8%)	122 (49.6%)	1.14 (0.85–1.53)	0.372	1.06 (0.80–1.41)	0.673
rs1419881	*TCF19*	C	361 (40.2%)	103 (45.6%)	126 (51.2%)	0.80 (0.60–1.08)	0.142	0.64 (0.48–0.85)	0.002
rs652888	*EHMT2*	C	329 (36.6%)	76 (33.6%)	84 (34.1%)	1.14 (0.84–1.55)	0.400	1.11 (0.83–1.50)	0.478

Abbreviation: CI, confidence interval; OR, odds ratio.
[a]Defined by using data from public database (NCBI).
[b]Defined as the combination between HCC and CHB.
doi:10.1371/journal.pone.0086007.t002