

Fig. 4 continued

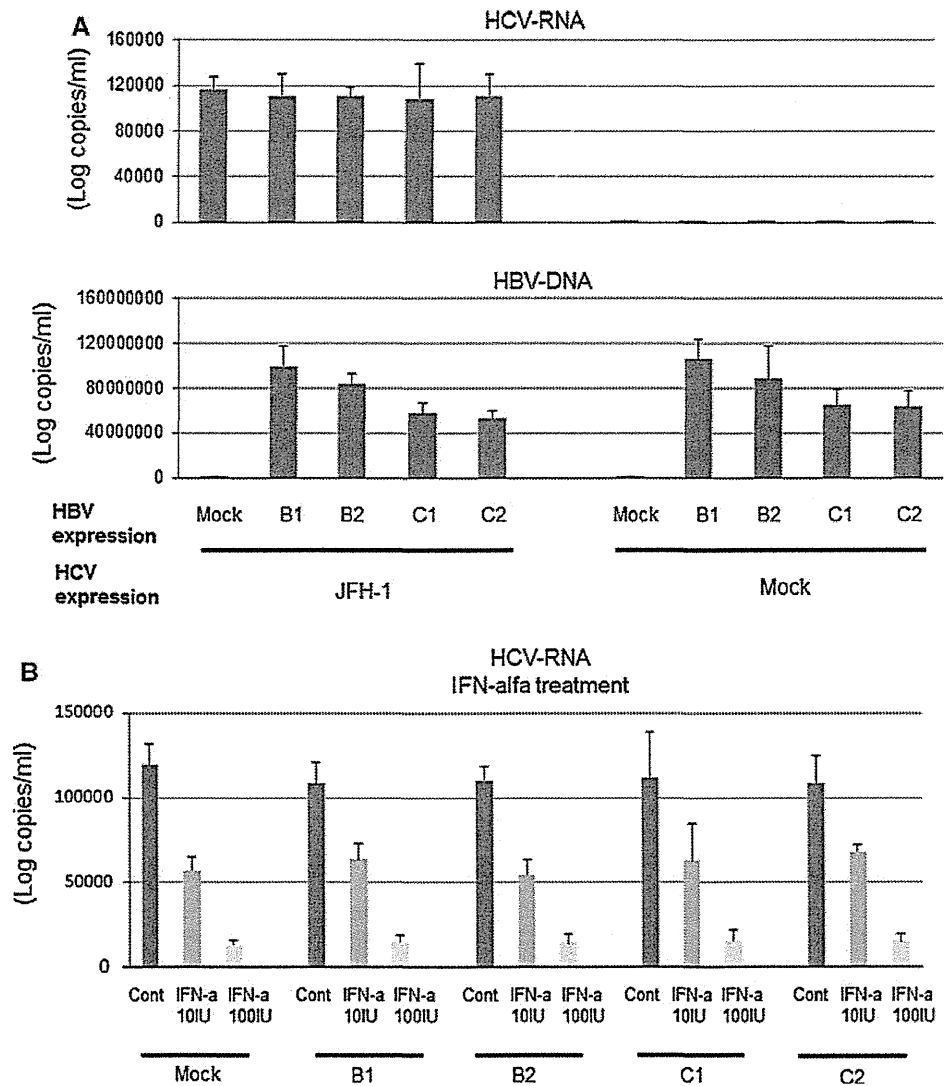
Discussion

The immunopathogenesis of dual hepatitis B and C infection is not clear, given the complexity of viral and host factors [19, 21, 48–50]. However, detailed understanding of specific patients with dual hepatitis B and C infection could contribute to improving the treatment and follow up of these patients. Therefore, we focused on two representative patients with HBV/HCV dual infection who received Peg-IFN/RBV therapy.

Concerning the virological results, patient A had genotype 1b, HCV-Core 70 wild-type and low mutation of ISDR HCV and genotype C HBV. It has been reported that genotype 1b HCV is common in Japan and is usually difficult to treat in comparison to genotypes 2a and 2b [51]. Among genotype 1b HCV strains, HCV-Core 70 wild-type HCV is easily decreased by Peg-IFN/RBV therapy [51]. On the other hand, it has been reported that in genotype 1b HCV low mutation of ISDR is difficult to treat [52]. Patient B had almost the same background of HCV—genotype 1b, HCV-Core 70 wild-type, and low mutation of ISDR—as patient A. However, the background of host factors that could affect the responsiveness of IFN-based therapy was

different between patients A and B. For example, patient A had a hetero allele of the IL-28B polymorphism, advanced fibrosis, and fatty changes of the liver. On the other hand, patient B had the major allele of the IL-28B polymorphism and mild fibrosis. Moreover, the background of HBV in patient B was completely different from that in patient A. It has been reported that HBV genotype Bj is usually more susceptible to IFN-based therapy than genotype C [45, 53]. Therefore, not only the HBV factors but also the combination of host factors and HBV factors might affect the responsiveness to IFN-based therapy. In patient A, the responsiveness of HCV during Peg-IFN/RBV therapy was relatively poor. However, the viral titers of HCV were lower than 1.2 log copies/ml at 7 months after the start of therapy. During the reduction of the HCV viral titers, the titers of HBV and HBeAg specific IL-10-secreting cells were gradually increased. Although patient A had received Peg-IFN/RBV therapy for up to 18 months, HCV-RNA increased again 12 months after the start of the therapy. The sustained Th1 immune suppression might have contributed to the relapse of HCV. Not only weak up-regulation of HCV-specific Th1 immune reaction but also strong up-regulation of HBV-specific IL-10-secreting activity was detected during Peg-IFN/RBV therapy in patient A [26, 35]. Moreover, increased HBeAg could be detected 9 months after the start of the therapy. Fluctuations of activated CD4 cells, CD8 cells, NK cells, and NK-T cells could be seen in patient A. On the other hand, in patient B, the responsiveness of HBV and HCV during Peg-IFN/RBV therapy was good. Moreover, the immune response of patient B was almost comparable to the responses in the patients with HCV mono-infection and those with HBV-genotype Bj mono-infection. Previously, it has been reported that Peg-IFN/RBV therapy could achieve almost the same SVR rates in patients with HCV/HBV dual infection and those with HCV mono-infection [54–56]. We assume that the results in these studies were obtained from patients similar to our patient B, because the number of patients with HCV-dominant infection is much higher than the number of those with HBV/HCV dual active infection such as our patient A. Patients with HBV/HCV dual active infection such as patient A are relatively rare in Japan. However, it is necessary to understand the immunopathogenesis of these patients, because Peg-IFN/RBV therapy might not be sufficient to eradicate or control HBV/HCV in these difficult-to-treat patients. One of the candidate therapies for such patients might be Entecavir (ETV)/Peg-IFN/RBV sequential therapy. The effect of HBV specific regulatory T cells might contribute to the immunosuppression of not only HBV but also HCV [35]. In some previous studies, including ours, it has been reported that HBV replication might contribute to immune suppression [19, 29].

Fig. 5 In vitro analysis of HBV/HCV dual infection. The titers of HCV-RNA and HBV-DNA are shown. *B1* indicates genotype Bj35 clone. *B2* indicates genotype Bj56 clone. *C1* indicates genotype C-AT clone. *C2* indicates genotype C-22 clone (a). The titers of HCV-RNA after the IFN- α treatment are shown (b)



In the present study, we employed an in vitro coinfection system to analyze the direct interaction between HBV and HCV. In our system, we used several different HBV clones, because it is necessary to consider the effects of different genotypes. Although we could not detect the direct interaction of HBV/HCV in our system, we could not exclude the possibility of indirect interaction between cytokines and chemokines produced from virus-infected hepatocytes. We are now analyzing the chemokines produced from hepatoma cells with different HBV genotype clones (ongoing study).

In conclusion, we analyzed data from representative patients with HBV/HCV dual infection sequentially and precisely. Because many different kinds of backgrounds might affect immunoreactions, we focused on representative patients and analyzed the immunological responses extensively. There might be a group of patients with very

difficult-to-treat dual infections. We need to understand the immunopathogenesis of such patients to develop the appropriate therapy.

Acknowledgments This work was supported in part by a Grant-in Aid from the Ministry of Education, Culture, Sport, Science, and Technology of Japan (Y.K. #23790761), and grants from the Ministry of Health, Labor, and Welfare of Japan.

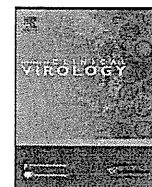
Conflict of interest The authors declare that they have no conflict of interest.

References

- Alter MJ, Kruszon-Moran D, Nainan OV, et al. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N Engl J Med.* 1999;341(8):556–62.

2. Tiollais P, Pourcel C, Dejean A. The hepatitis B virus. *Nature*. 1985;317(6037):489–95.
3. Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. *Lancet*. 2003;362(9401):2089–94.
4. Poynard T, Yuen MF, Ratziu V, Lai CL. Viral hepatitis C. *Lancet*. 2003;362(9401):2095–100.
5. Potthoff A, Manns MP, Wedemeyer H. Treatment of HBV/HCV coinfection. *Expert Opin Pharmacother*. 2010;11(6):919–28.
6. Chu CJ, Lee SD. Hepatitis B virus/hepatitis C virus coinfection: epidemiology, clinical features, viral interactions and treatment. *J Gastroenterol Hepatol*. 2008;23(4):512–20.
7. Bini EJ, Perumalswami PV. Hepatitis B virus infection among American patients with chronic hepatitis C virus infection: prevalence, racial/ethnic differences, and viral interactions. *Hepatology*. 2010;51(3):759–66.
8. Halima SB, Bahri O, Maamouri N, et al. Serological and molecular expression of hepatitis B infection in patients with chronic hepatitis C from Tunisia, North Africa. *Virology*. 2010;7:229.
9. Saravanan S, Velu V, Nandakumar S, et al. Hepatitis B virus and hepatitis C virus dual infection among patients with chronic liver disease. *J Microbiol Immunol Infect*. 2009;42(2):122–8.
10. Lee LP, Dai CY, Chuang WL, et al. Comparison of liver histopathology between chronic hepatitis C patients and chronic hepatitis B and C-coinfected patients. *J Gastroenterol Hepatol*. 2007;22(4):515–7.
11. Liu Z, Hou J. Hepatitis B virus (HBV) and hepatitis C virus (HCV) dual infection. *Int J Med Sci*. 2006;3(2):57–62.
12. Tsai JF, Jeng JE, Ho MS, et al. Effect of hepatitis C and B virus infection on risk of hepatocellular carcinoma: a prospective study. *Br J Cancer*. 1997;76(7):968–74.
13. Cho LY, Yang JJ, Ko KP, et al. Coinfection of hepatitis B and C viruses and risk of hepatocellular carcinoma: systematic review and meta-analysis. *Int J Cancer*. 2011;128(1):176–84.
14. Huo TI, Huang YH, Hsia CY, et al. Characteristics and outcome of patients with dual hepatitis B and C-associated hepatocellular carcinoma: are they different from patients with single virus infection? *Liver Int*. 2009;29(5):767–73.
15. Kew MC. Interaction between hepatitis B and C viruses in hepatocellular carcinogenesis. *J Viral Hepat*. 2006;13(3):145–9.
16. Liu CJ, Chen PJ, Chen DS. Dual chronic hepatitis B virus and hepatitis C virus infection. *Hepatol Int*. 2009;3(4):517–25.
17. Bellecave P, Gouttenoire J, Gajer M, et al. Hepatitis B and C virus coinfection: a novel model system reveals the absence of direct viral interference. *Hepatology*. 2009;50(1):46–55.
18. Eyre NS, Phillips RJ, Bowden S, et al. Hepatitis B virus and hepatitis C virus interaction in Huh-7 cells. *J Hepatol*. 2009;51(3):446–57.
19. Chisari FV, Ferrari C. Hepatitis B virus immunopathogenesis. *Annu Rev Immunol*. 1995;13:29–60.
20. Koziel MJ. The role of immune responses in the pathogenesis of hepatitis C virus infection. *J Viral Hepat*. 1997;4(Suppl 2):31–41.
21. Rice CM, Walker CM. Hepatitis C virus-specific T lymphocyte responses. *Curr Opin Immunol*. 1995;7(4):532–8.
22. Nan XP, Zhang Y, Yu HT, et al. Circulating CD4⁺CD25^{high} regulatory T cells and expression of PD-1 and BTLA on CD4⁺ T cells in patients with chronic hepatitis B virus infection. *Viral Immunol*. 2010;23(1):63–70.
23. Kondo Y, Ueno Y, Shimosegawa T. Immunopathogenesis of hepatitis B persistent infection: implications for immunotherapeutic strategies. *Clin J Gastroenterol*. 2009;2(2):71–9.
24. Peng G, Li S, Wu W, Sun Z, Chen Y, Chen Z. Circulating CD4⁺CD25⁺ regulatory T cells correlate with chronic hepatitis B infection. *Immunology*. 2008;123(1):57–65.
25. Maier H, Isogawa M, Freeman GJ, Chisari FV. PD-1:PD-L1 interactions contribute to the functional suppression of virus-specific CD8⁺ T lymphocytes in the liver. *J Immunol*. 2007;178(5):2714–20.
26. Kondo Y, Kobayashi K, Ueno Y, et al. Mechanism of T cell hyporesponsiveness to HBcAg is associated with regulatory T cells in chronic hepatitis B. *World J Gastroenterol*. 2006;12(27):4310–7.
27. Stoop JN, van der Molen RG, Baan CC, et al. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology*. 2005;41(4):771–8.
28. Kondo Y, Kobayashi K, Asabe S, et al. Vigorous response of cytotoxic T lymphocytes associated with systemic activation of CD8 T lymphocytes in fulminant hepatitis B. *Liver Int*. 2004;24(6):561–7.
29. Kondo Y, Asabe S, Kobayashi K, et al. Recovery of functional cytotoxic T lymphocytes during lamivudine therapy by acquiring multi-specificity. *J Med Virol*. 2004;74(3):425–33.
30. Kondo Y, Ueno Y, Kakazu E, et al. Lymphotropic HCV strain can infect human primary naive CD4(+) cells and affect their proliferation and IFN-gamma secretion activity. *J Gastroenterol*. 2011;46:232–41.
31. Kondo Y, Machida K, Liu HM, et al. Hepatitis C virus infection of T cells inhibits proliferation and enhances fas-mediated apoptosis by down-regulating the expression of CD44 splicing variant 6. *J Infect Dis*. 2009;199(5):726–36.
32. Kondo Y, Sung VM, Machida K, Liu M, Lai MM. Hepatitis C virus infects T cells and affects interferon-gamma signaling in T cell lines. *Virology*. 2007;361(1):161–73.
33. Ulsenheimer A, Gerlach JT, Gruener NH, et al. Detection of functionally altered hepatitis C virus-specific CD4 T cells in acute and chronic hepatitis C. *Hepatology*. 2003;37(5):1189–98.
34. Cramp ME, Rossol S, Chokshi S, Carucci P, Williams R, Naoumov NV. Hepatitis C virus-specific T-cell reactivity during interferon and ribavirin treatment in chronic hepatitis C. *Gastroenterology*. 2000;118(2):346–55.
35. Kondo Y, Ueno Y, Kobayashi K, et al. Hepatitis B virus replication could enhance regulatory T cell activity by producing soluble heat shock protein 60 from hepatocytes. *J Infect Dis*. 2010;202(2):202–13.
36. Nguyen LH, Ko S, Wong SS, et al. Ethnic differences in viral dominance patterns in patients with hepatitis B virus and hepatitis C virus dual infection. *Hepatology*. 2011;53(6):1839–45.
37. Takahashi M, Nishizawa T, Gotanda Y, et al. High prevalence of antibodies to hepatitis A and E viruses and viremia of hepatitis B, C, and D viruses among apparently healthy populations in Mongolia. *Clin Diagn Lab Immunol*. 2004;11(2):392–8.
38. Ina Y. ODN: a program package for molecular evolutionary analysis and database search of DNA and amino acid sequences. *Comput Appl Biosci*. 1994;10(1):11–2.
39. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res*. 1994;22(22):4673–80.
40. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol*. 1987;4(4):406–25.
41. Felsenstein J. Estimating effective population size from samples of sequences: a bootstrap Monte Carlo integration method. *Genet Res*. 1992;60(3):209–20.
42. Perriere G, Gouy M. WWW-query: an on-line retrieval system for biological sequence banks. *Biochimie*. 1996;78(5):364–9.
43. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med*. 2005;11(7):791–6.
44. Takeuchi T, Katsume A, Tanaka T, et al. Real-time detection system for quantification of hepatitis C virus genome. *Gastroenterology*. 1999;116(3):636–42.

45. Sugauchi F, Kumada H, Sakugawa H, et al. Two subtypes of genotype B (Ba and Bj) of hepatitis B virus in Japan. *Clin Infect Dis*. 2004;38(9):1222–8.
46. Sugauchi F, Orito E, Ichida T, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. *Gastroenterology*. 2003;124(4):925–32.
47. Orito E, Mizokami M, Sakugawa H, et al. A case–control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology*. 2001;33(1):218–23.
48. Raimondo G, Brunetto MR, Pontisso P, et al. Longitudinal evaluation reveals a complex spectrum of virological profiles in hepatitis B virus/hepatitis C virus-coinfected patients. *Hepatology*. 2006;43(1):100–7.
49. Chuang WL, Dai CY, Chang WY, et al. Viral interaction and responses in chronic hepatitis C and B coinfecting patients with interferon-alpha plus ribavirin combination therapy. *Antivir Ther*. 2005;10(1):125–33.
50. Tsai SL, Liaw YF, Yeh CT, Chu CM, Kuo GC. Cellular immune responses in patients with dual infection of hepatitis B and C viruses: dominant role of hepatitis C virus. *Hepatology*. 1995;21(4):908–12.
51. Akuta N, Suzuki F, Hirakawa M, et al. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. *Hepatology*. 2010;52(2):421–9.
52. Fukuma T, Enomoto N, Marumo F, Sato C. Mutations in the interferon-sensitivity determining region of hepatitis C virus and transcriptional activity of the nonstructural region 5A protein. *Hepatology*. 1998;28(4):1147–53.
53. Akuta N, Kumada H. Influence of hepatitis B virus genotypes on the response to antiviral therapies. *J Antimicrob Chemother*. 2005;55(2):139–42.
54. Yu ML, Lee CM, Chuang WL, et al. HBsAg profiles in patients receiving peginterferon alfa-2a plus ribavirin for the treatment of dual chronic infection with hepatitis B and C viruses. *J Infect Dis*. 2010;202(1):86–92.
55. Yu JW, Sun LJ, Zhao YH, Kang P, Gao J, Li SC. Analysis of the efficacy of treatment with peginterferon alpha-2a and ribavirin in patients coinfecting with hepatitis B virus and hepatitis C virus. *Liver Int*. 2009;29(10):1485–93.
56. Liu CJ, Chuang WL, Lee CM, et al. Peginterferon alfa-2a plus ribavirin for the treatment of dual chronic infection with hepatitis B and C viruses. *Gastroenterology*. 2009;136(2):496.e3–504.e3.



Association between S21 substitution in the core protein of hepatitis B virus and fulminant hepatitis

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ARTICLE INFO

Article history:

Received 15 February 2012

Received in revised form 11 June 2012

Accepted 20 June 2012

Keywords:

HBV

FHB

Genotype

T1961V

C1962D

ABSTRACT

Background: The viral factors of hepatitis B virus (HBV), such as genotypes and mutations, were reported to affect the development of fulminant hepatitis B (FHB), but the mechanism is still unclear.

Objectives: To investigate HBV mutations associated with FHB, especially in the subgenotype B1/Bj HBV (HBV/B1), which are known to cause FHB frequently in Japan.

Study design: A total of 96 serum samples from acute self-limited hepatitis B (AHB) patients and 13 samples from FHB patients were used for full-genome/partial sequencing. A total of 107 chronic infection patients with HBV were also examined for the distribution of mutants.

Results: In the analysis of full-genome sequences of HBV/B1 (FHB, $n = 11$; non-FHB, $n = 35$) including those from the databases, mutations at nt 1961 [T1961V (not T)] and nt 1962 [C1962D (not C)], which change S21 in the core protein, were found more frequently in FHB than in non-FHB (100% vs. 20%, 55% vs. 3%, respectively). When our FHB and AHB samples were compared, T1961V and C1962D were significantly more frequent in FHB than in AHB, both in the overall analysis (46% vs. 6%, 39% vs. 3%, respectively) and in HBV/B1 (100% vs. 29%, 100% vs. 14%, respectively). A newly developed PCR system detecting T1961V showed that HBV/B1 and low viral load were independent factors for the mutation among chronic infection patients.

Conclusions: T1961V/C1962D mutations were found frequently in FHB, especially in HBV/B1. The resulting S21 substitution in the core protein may play important roles in the development of FHB.

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1. Background

Hepatitis B virus (HBV) infection is one of the most common viral diseases affecting humans. HBV causes a spectrum of liver

diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.¹ Patients with acute HBV infection sometimes develop fulminant hepatitis B (FHB). The survival rate of FHB patients is still low² and they require careful management including liver transplantation.³

HBV contains a small (3.2 kb), circular, partially double-stranded DNA genome.¹ Based on the genomic variability noted among HBV isolates, HBV sequences have been classified into at least 8 genotypes (A–H), and recently, additional 2 genotypes (I and J) were proposed tentatively.^{4,5} The genotypes/subgenotypes of HBV have been known to affect the disease outcome.^{6–8} As for FHB, the subgenotype B1/Bj HBV (HBV/B1) was reported to be more often associated than subgenotypes B2/Ba, genotype A, and genotype

Abbreviations: AHB, acute self-limited hepatitis B; CTL, cytotoxic T lymphocyte; FHB, fulminant hepatitis B; HBV, hepatitis B virus; HBV/B1, subgenotype B1/Bj HBV; HBV/C2, subgenotype C2/Ce HBV; SSP, sequence-specific primer.

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C.^{6,9} Although HBV genotypes are classified by a sequence divergence of more than 8% in the entire genome,¹⁰ little is known about which region or nucleotide substitutions of the genome are responsible for the disease outcome differences among genotypes.

Several mutations in the precore region such as G1896A,^{6,9,11–15} which makes a stop codon and abrogates HBeAg, and those in the core promoter such as double mutations of A1762T/G1764A^{6,9,16} were reported to be associated with FHB. Also, T1753V [A, C or G (not T)],¹⁴ T1754V,¹⁴ G1899A,⁹ and A2339G⁹ were found in FHB patients more frequently than in AHB patients. Some of them were described as having the ability to enhance the replication capacity of HBV in vitro.^{6,17–19} Recently, we reported that insertions/deletions in the precore region, which cause a frameshift of the precore protein and abrogates HBeAg, was found frequently in FHB patients with HBV/B1 and enhanced the replication capacity of HBV in vitro.¹⁵ However, some conflicting results have been described^{20,21} and it is considered that there are still unknown mechanisms in regard to the pathogenesis of FHB.

2. Objectives

Here we studied HBV mutations that were associated with FHB by analyzing full-genome sequences. Next, additional patients with acute and chronic HBV infection were analyzed for clarifying prevalence of mutations.

3. Study design

3.1. Serum samples

A total of 109 serum samples from patients with acute HBV infection including 13 FHB patients and a total of 107 serum samples from patients with chronic HBV infection were obtained from March 1998 to August 2011 in Tohoku University Hospital and other 5 hospitals in the same area. The diagnosis of acute infection was made based on the detection of high-titered immunoglobulin M anti-hepatitis core without any history of prior liver diseases. There were no acute infection patients who retained HBsAg for more than 6 months. The diagnosis of fulminant hepatitis was made based on a slightly modified definition in Inuyama Symposium (Aichi, Japan, 1981) of the original definition²²: coma grade II or higher, and a prothrombin time of less than 40% developing within 8 weeks after the onset.

3.2. Amplification of HBV sequences and determination of genotypes

To determine the full-genome sequences of HBV, serum samples from 10 of 13 FHB patients were used. Because the sample volume of the remaining 3 samples was relatively small, they were used only for partial sequencing. Additionally, a serum sample from HBV carrier who developed FHB was studied only for full-genome comparison. The same samples that were used for full-genome sequencing in this study had been used also in a previous report to determine only partial sequences.¹⁵ Amplification of the entire HBV genome was performed by methods essentially similar to those described previously.²³ To amplify the 548-nt sequence in the core promoter/precore/core region [nt 1719–2266 (excluding primer sequences), the nucleotide numbers are in accordance with an HBV/B1 isolate of 3215 nt (D00329)], the DNA solution was subjected to nested PCR. The first round PCR was carried out with primers B053 (Table S1) and B055, and the second round was carried out with primers B054 and B052. The amplification products were sequenced on both strands directly using the BigDye Terminator v3.1 Cycle Sequencing kit on an ABI PRISM 3100 Genetic

Analyzer (Applied Biosystems, Foster City, CA). The AHB/FHB samples used for partial sequencing had never used in the previous study.

For patients with chronic HBV infection, an HBsAg subtype EIA kit (Institute of Immunology, Tokyo, Japan) was used to determine the genotypes of HBV. Only genotype B samples were subjected to direct sequencing for subgenotyping.

3.3. PCR with sequence-specific primers detecting the mutation at nt 1961

To detect mutations at nt 1961 (T1961V) rapidly without the sequencing analysis, a PCR system with sequence-specific primers (SSP) was newly developed in this study. Because the sequences around nt 1961 were relatively conserved, two opposite primers, B050 and B051, whose 3' ends are located at nt 1961 could be designed. B050 can anneal only with T1961V mutants to make 349 bp products. B051 can detect sequences without T1961V and make 208 bp products. The DNA solution was subjected to PCR with primers 8 pmol of B049, B051, and B052, and 64 pmol of B050, and AmpliTaq Gold PCR Master Mix (Applied Biosystems) in 20 μ l reaction. The PCR was carried out for 40 cycles (95 °C for 15 s, 60 °C for 15 s, 72 °C for 60 s). If both 208 bp and 349 bp products were amplified, the HBV was considered to have the mix type mutations at nt 1961.

3.4. Statistical analysis

Statistical analyses were performed using Fisher's exact probability test or the chi-square test for comparison of proportions between two groups and Mann–Whitney *U* test for comparison of continuous variables between two groups. Multivariate analyses were performed with a logistic regression model using SPSS Statistics 19 (IBM, Armonk, NY).

4. Results

4.1. Full-genome comparative analysis of HBV

Of 10 full-genome sequences from FHB patients, 4 sequences were revealed to belong to HBV/B1 and 5 sequences were subgenotype C2/Ce (HBV/C2), and one sequence was subgenotype B2/Ba. A sequence from an HBV carrier who developed FHB (BFJT2009-1) was HBV/B1. A phylogenetic tree was constructed based on the full-genome sequences of genotype A–J HBV (Fig. S1) and the sequence data have been assigned to the GenBank/EMBL/DBJ with the accession numbers AB642091–AB642101.

In August 2011, a total of 41 full-genome sequences of HBV/B1 could be retrieved from Hepatitis Virus Database²⁴ and GenBank/EMBL/DBJ. Of them, 6 were obtained from FHB patients, and 35 were others including chronically infected patients. First, a consensus sequence of HBV/B1 was deduced from the 35 non-FHB strains. Then the numbers of nucleotide mutations, which were different from the consensus sequence, in all of the 3215 nt of the HBV/B1 genome were compared between FHB ($n = 11$, including 5 sequences determined in this study) and non-FHB. When the *p*-values of mutations were calculated in each nucleotide, only nt 1961 and 1962 had lower *p*-values than 1.0×10^{-3} (Fig. 1A). The full-genome sequences of HBV/C2 were also compared between FHB ($n = 15$, including 5 sequences determined in this study) and non-FHB ($n = 35$) in the same way (Fig. 1B). However, such a low *p*-value was not found in the HBV/C2 strains. Table S2 shows the nucleotides that had lower *p*-values than 1.0×10^{-2} . The partial nucleotide sequences of HBV/B1 around nt 1961/1962 are shown in Fig. 1C. All of the 11 FHB strains had the mutation at nt 1961 and 6 strains had the mutation at nt 1962. Although the mutations at nt

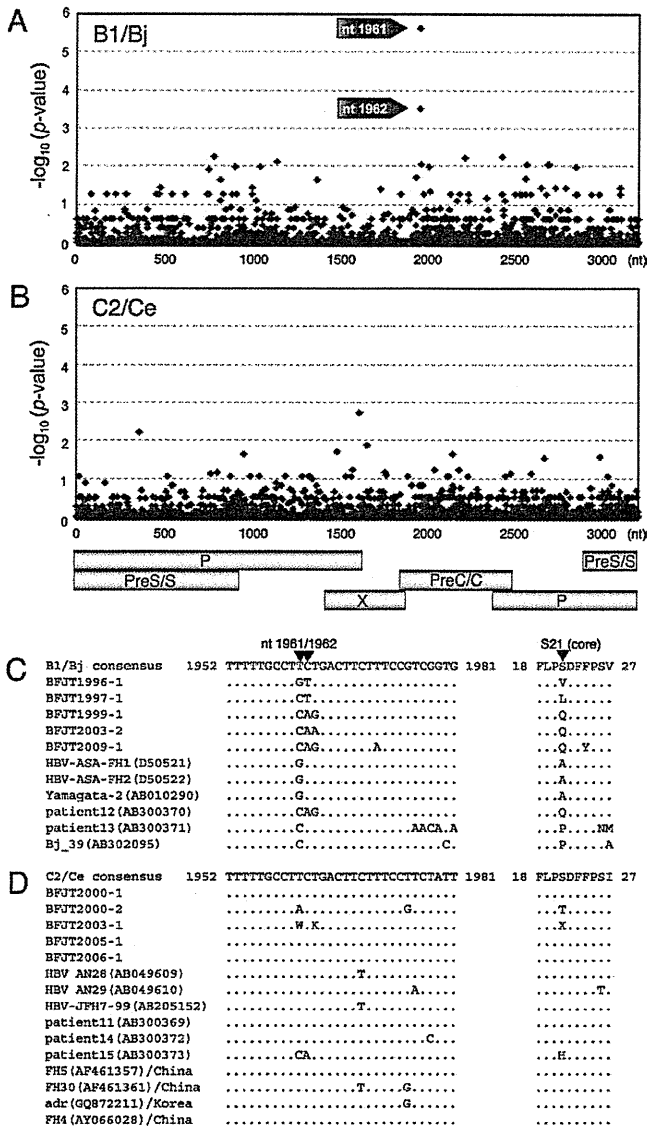


Fig. 1. Comparison of HBV full-genome sequences between FHB and non-FHB. (A) Comparison of HBV/B1 between FHB ($n=11$) and non-FHB ($n=35$). The frequencies of mutations at each nucleotide were analyzed, and the reciprocal numbers of p values were plotted. (B) Comparison of HBV/C2 between FHB ($n=15$) and non-FHB ($n=35$). (C) Partial nucleotide sequences of HBV/B1 around nt 1961/1962 and deduced amino acid sequences of FHB strains. The consensus sequences deduced from non-FHB sequences are indicated at the top. (D) Sequences of HBV/C2. W (nucleotide), T or A; X (amino acid), S or T.

1961/1962 were minor in HBV/C2 (Fig. 1D), 3/15 (20%) of FHB and 2/35 (6%) of non-FHB had the mutations. These mutations could change the amino acid of S21 in the core protein (Fig. 1C and D), which is located in an HLA-A2 restricted cytotoxic T lymphocyte (CTL) epitope of HBeAg18–27.²⁵

4.2. Distribution of S21 substitution among patients with HBV acute infection

Next we compared partial sequences containing nt 1961/1962 using our acute self-limited hepatitis B (AHB, $n=96$) and FHB ($n=13$) samples. The clinical characteristics of FHB/AHB patients are shown in Table 1. Consistent with previous reports,^{6,9} the HBeAg positive rate was significantly lower in FHB patients. As reported previously from Japan, HBV/C2 was most prevalent

Table 1
Clinical characteristics of patients with FHB and AHB in this study.

	FHB ($n=13$) ^a	AHB ($n=96$) ^a	p -Value
Age	45.2 ± 16.0	32.5 ± 13.1	0.004
Male	11 (84.6)	59 (61.5)	0.088
HBeAg positive	1 (7.7)	61 (67.8)	<0.0005
Peak T. Bil (mg/dl) ^b	15.1 ± 8.8	9.0 ± 9.0	0.096
Peak AST (IU/l) ^b	4663 ± 3786	1451 ± 1686	0.074
Peak ALT (IU/l) ^b	4841 ± 3850	2350 ± 1735	0.300
Lowest PT (%) ^b	19.7 ± 11.6	73.7 ± 23.8	<0.0005
HBV DNA (log copies/ml) ^c	6.7 ± 2.2	6.7 ± 1.9	0.954
HBV subgenotype			
A2/Ae	0 (0)	19 (19.8)	0.070
B1/Bj	4 (30.8)	14 (14.6)	0.141
B2/Ba	2 (15.4)	5 (5.2)	0.196
B7	0 (0)	1 (1.0)	0.238
C1/Cs	0 (0)	3 (3.1)	0.681
C2/Ce	7 (53.8)	54 (56.3)	0.680
Therapy			
Liver transplantation	3 (23.1)	0 (0)	0.001
Nucleoside analog ^d	4 (38.5)	9 (9.4)	0.048
Deceased	6 (46.2)	0 (0)	<0.0001

T. Bil, total bilirubin; AST, aspartate aminotransferase; ALT, alanine aminotransferase; PT, prothrombin time.

^a Values are expressed as the mean ± standard deviation or numbers of patients (%).

^b Data of 28 patients could not be obtained.

^c Data of 8 patients could not be obtained.

^d Lamivudine or entecavir was used.

(61/109, 56%). HBV/B1 was found more frequently (18/109, 17%) than the reports of all Japan (7%)²⁶ and was reported to be a characteristic of northeast Japan.^{7,15}

Besides the mutations at nt 1961 and 1962, several mutations that were reported to be associated with FHB^{6,9,11–14,16} were compared. The frameshift insertions/deletions in the precore region that we reported recently¹⁵ were also included. In the overall analysis, the mutations of G1896A, T1961V, and C1962D [A, G or T (not C)] were found much more frequently in FHB patients than in AHB patients (62% vs. 8%, $p=3.0 \times 10^{-5}$; 46% vs. 6%, $p=5.4 \times 10^{-4}$; 39% vs. 3%, $p=5.1 \times 10^{-4}$, respectively) (Fig. 2A). The mutations of A1762T/T1764A and the precore frameshift were significantly more frequent in patients with FHB (48% vs. 16%, $p=0.018$; 23% vs. 1%, $p=0.0051$), as well. Notably, when HBV/B1 isolates were compared, the frequencies of only T1961V, C1962D, and the precore frameshift were significantly different (100% vs. 29%, $p=0.023$; 100% vs. 14%, $p=0.0049$; 75% vs. 7% $p=0.019$) (Fig. 2B). In the analysis of HBV/C2 isolates, G1896A (71% vs. 7%, $p=3.9 \times 10^{-4}$), A1762T/G1764A (71% vs. 26%, $p=0.026$), and T1753V (43% vs. 9%, $p=0.042$) were significantly more frequent in FHB patients (Fig. 2C), different from HBV/B1.

Additionally, we analyzed the T1961V frequency in combination with the major mutations G1896A and A1762T/G1764A. In the overall analysis, the frequency of isolates without these mutations was significantly higher in AHB than FHB (77% vs. 8%, $p=1.8 \times 10^{-6}$) (Fig. 2D). The combinations of A1762T/G1764A and G1896A, and all three mutations were found more frequently in FHB patients than in AHB patients (31% vs. 3%, $p=0.0036$; 15% vs. 1%, $p=0.037$, respectively). When analyzed separately 2 subgenotypes, the distribution of combination pattern in FHB patients was quite different (Fig. 2E and F). Whereas T1961V was present alone (50%) or with G1896A (50%) in HBV/B1, all HBV/C2 isolates with T1961V had both A1762T/G1764A and G1896A. Because the all HBV/B1 isolates only with T1961V had the precore frameshift, which abrogates HBeAg like as G1896A,¹⁵ T1961V seemed to be related to HBeAg-defective strains.

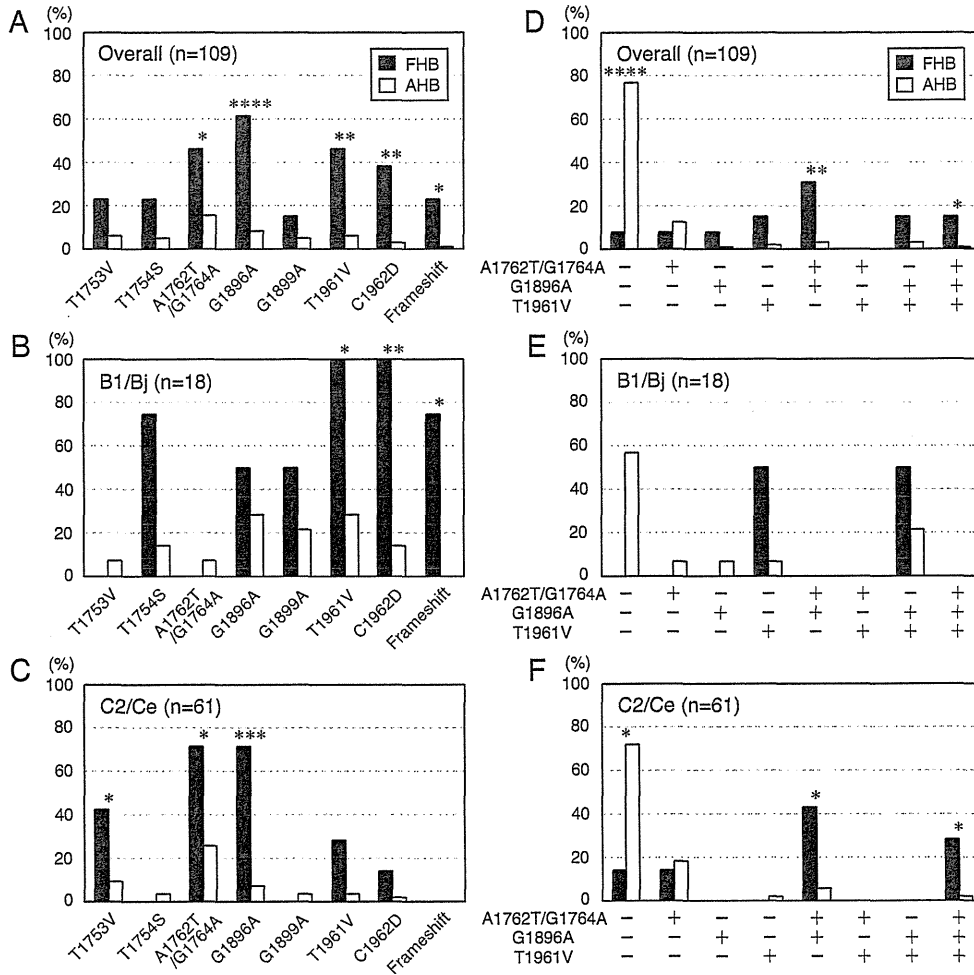


Fig. 2. Comparison of mutations between FHB and AHB in the core promoter/precore/core region. Besides T1961V and C1962D, several mutations that had been reported to be associated with FHB were also included. (A) Comparison of the overall HBV sequences between FHB (n=13) and AHB (n=96). (B) Comparison of HBV/B1 between FHB (n=4) and AHB (n=14). (C) Comparison of HBV/C2 between FHB (n=7) and AHB (n=54). D=A, G or T (not C); S=C or G; V=A, C or G (not T); *p < 0.05; **p < 0.005; ***p < 0.0005; ****p < 0.00005.

4.3. Distribution of T1961V in chronically HBV-infected patients

A PCR system with SSP was established in this study to detect T1961V more easily (Fig. 3). Using the PCR system, the frequency of T1961V was evaluated using 107 chronically HBV-infected patients [mean age, 50.6 ± 13.8; male, 73 (68.2%); HBeAg positive, 49 (45.8%); mean ALT (IU/l), 142.9 ± 177.7; mean HBV DNA (log copies/ml), 6.4 ± 1.7; liver cirrhosis, 27 (25.2%); hepatocellular carcinoma, 20 (18.7%)]. Of them, 103 (96%) could be determined to be a wild type or a mutant at nt 1961. The remaining 4 patients showed only 512 bp products. Sequencing analysis showed that HBV of the 4 patients had minor mutations within the B051-annealing site and no mutation at nt 1961. In total, 18/107 (17%) patients had T1961V mutants including the mixed type (Table 2). Of note, the frequency of T1961V including the mixed type was significantly higher in HBeAg-negative patients than in HBeAg-positive patients (29% vs. 2%, p = 1.7 × 10⁻⁴). Patients with low HBV DNA levels (<6.6 log copies/ml) in the serum had T1961V mutants more frequently than those with high levels (31% vs. 4%, p = 1.8 × 10⁻⁴). Although patients treated with nucleos(t)ide analogs had T1961V mutants frequently, it may be a result from the fact that HBeAg-negative patients with low HBV DNA are rarely treated with nucleos(t)ide analogs. When the HBV genotypes were

compared, the T1961V mutants were detected more frequently in HBV/B1 than in the others (41% vs. 11%, p = 0.002). HLA-A2 positivity, which was detected as described previously,^{27,28} was not associated with the mutation significantly. A multivariate analysis showed that the HBV/B1 (odds ratio 7.635, p = 0.006) and HBV DNA levels (odds ratio 0.548, p = 0.009) were independent factors for the T1961V mutation.

5. Discussion

In this study, we found first that the S21 substitution in the core protein due to T1961V (±C1962D) was associated with FHB, especially in HBV/B1. Because the first stage analysis of HBV full-genome sequences included HBV strains from chronically infected patients in the databases, mutations that were common in chronic infection such as G1896A²⁹ and A1762T/G1764A³⁰ were not detected. In the analysis, only mutations at nt 1961 and 1962 in HBV/B1 were picked up with lower p-values than 1.0 × 10⁻³, and we could confirm that these mutations were more frequent in FHB patients in the second stage analysis of our acutely infected patients. Therefore, the rapid examination of T1961V by PCR with SSP in this study was considered to be useful to predict the development of FHB in acutely infected patients.

Table 2
Distribution of the mutation at nt 1961 among HBV chronically infected patients detected by PCR with SSP.

	n	Wild type (%)	Mixed type (%)	Mutant (%)	p-Value ^a
Overall	107	89 (83.2)	6 (5.6)	12 (11.2)	
Age (years)					0.245
<53	56	49 (87.5)	1 (1.8)	6 (10.7)	
≥53	51	40 (78.4)	5 (9.8)	6 (11.8)	
HBeAg					<0.0005
Positive	49	48 (98.0)	0 (0)	1 (2.0)	
Negative	58	41 (70.7)	6 (10.3)	11 (19.0)	
HBV DNA (log copies/ml)					<0.0005
<6.6	52	36 (69.2)	6 (11.5)	10 (19.2)	
≥6.6	55	53 (96.4)	0 (0)	2 (3.6)	
Liver cirrhosis					0.496
Present	27	22 (81.5)	3 (11.1)	2 (7.4)	
Absent	80	67 (83.8)	3 (3.8)	10 (12.5)	
Hepatocellular carcinoma					0.083
Present	20	14 (70.0)	4 (20.0)	2 (10.0)	
Absent	87	75 (86.2)	2 (2.3)	10 (11.5)	
Treatment					0.049
Nucleos(t)ide analog ^b	24	23 (95.8)	0 (0)	1 (4.2)	
Naïve	83	66 (79.5)	6 (7.2)	11 (13.3)	
Genotypes					
A	4	3 (75.0)	1 (25.0)	0 (0)	
B	25	16 (64.0)	4 (16.0)	5 (20.0)	0.006 ^c
B1/Bj	22	13 (59.1)	4 (18.2)	5 (22.7)	0.002 ^c
B2/Ba	3	3 (100)	0 (0)	0 (0)	
C	78	70 (89.7)	1 (1.3)	7 (9.0)	
HLA-A2					0.056
Positive	32	30 (93.8)	0 (0)	2 (6.3)	
Negative	36	29 (80.6)	2 (5.6)	5 (13.9)	

^a Wild type vs. mixed type and mutant.
^b Twenty patients were treated with lamivudine, and 4 patients were treated with combination of lamivudine and adefovir dipivoxil. They were treated for more than 3 months.
^c Genotype B or subgenotype B1/Bj vs. others.

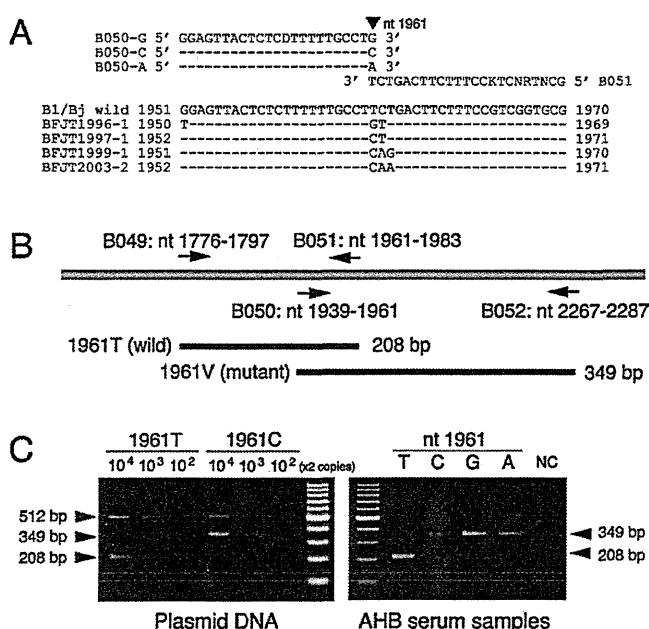


Fig. 3. Schema of the PCR with SSP established to detect the S21 mutation. (A) Primer sequences used in the PCR and corresponding sequences of the HBV/B1 wild type and 4 HBV/B1 FHB strains. Because the S21 mutants always had T1961V in our analysis, SSP was designed to anneal with either T1961V mutant or wild type. (B) Schema of the positions of primers used in the PCR, and the size of the resulting PCR products. T1961V mutants were distinguished by the difference of amplified products (wild type, 208 bp; mutant, 349 bp). (C) Patterns of the electrophoresis of the PCR products. The left panel shows the products amplified from the plasmid DNA with 1961T and 1961C of 2.0×10^4 to 2.0×10^2 copies, and the right panel shows the products amplified from DNA of AHB patient sera whose partial HBV sequences including nt 1961 were determined. The 512 bp products amplified by primers of B049 and B052 were not necessarily detected.

nt 1961–1963 of HBV encodes S21 in the core protein, which is located within an HLA-A2 restricted CTL epitope of HBeAg18–27,²⁵ and T1961V ± C1962D changes S21 to various amino acids. Interestingly, this mutant epitope had been suggested to act as T-cell receptor antagonist leading to inhibition of the CTL response to the wild type CTL epitope,³¹ and the S21 substitution was considered to be a CTL escape mutation.³² However, of our 3 FHB patients whose HLA-A types were known, 2 patients with S21 substitution did not have HLA-A2 (data not shown). Therefore the pathogenesis might not be necessarily due to the immune escape. Also, in patients with chronic infection, there were several HLA-A2-negative patients with T1961V mutants. The association between S21 substitution and HLA types has to be clarified further both in acutely infected patients and in chronically infected patients.

Because this study was limited by the small number of FHB patients and the cross-sectional nature, causal relationship could not be established well. While it is possible that S21 substitution predisposes acute infection patients to FHB, immune pressure during active hepatitis can also induce mutations. The latter possibility is less likely because of the short duration from infection, but further mechanistic study is needed to address the issue.

Through HBeAg seroconversion, which is an important event in chronic HBV infection, the HBV specific CTL repertoire develops, and mutations in the core gene increase.³³ It was reported that the S21 substitution was more frequent in HBeAg-negative chronic hepatitis B patients with low levels of HBV DNA than in those with high levels of HBV DNA,³² which is concordant with our results. A study from New Zealand reported that, in the core protein, S21 was one of the amino acids under significant positive selection pressure.³⁴ These data suggested that the S21 substitution favors the persistence of HBV in the late stage of the natural course of HBV chronic infection, despite the developed immunity.

In conclusion, the S21 substitution in the core protein due to T1961V ± C1962D was found more frequently in FHB patients,

especially those with HBV/B1, than in AHB patients or chronically infected patients. This finding may give an insight into the mechanism of FHB and may be useful to predict the development of FHB in acute HBV infection.

Funding

This work was supported by Grant-in-Aid for Young Scientists (B) (assignment no. 22790627) from Ministry of Education, Culture, Sports, Science, and Technology of Japan, and grants from Ministry of Health, Labor, and Welfare of Japan.

Competing interests

None.

Ethical approval

Not required.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jcv.2012.06.011>.

References

- Liang TJ. Hepatitis B: the virus and disease. *Hepatology* 2009;**49**(5):S13–21.
- Oketani M, Ido A, Tsubouchi H. Changing etiologies and outcomes of acute liver failure: a perspective from Japan. *J Gastroenterol Hepatol* 2011;**26**(Suppl 1):65–71.
- Hoofnagle JH, Carithers Jr RL, Shapiro C, Ascher N. Fulminant hepatic failure: summary of a workshop. *Hepatology* 1995;**21**(1):240–52.
- Olinger CM, Jutavijittum P, Hubschen JM, Yousukh A, Samountray B, Thammavong T, et al. Possible new hepatitis B virus genotype, southeast Asia. *Emerg Infect Dis* 2008;**14**(11):1777–80.
- Tatematsu K, Tanaka Y, Kurbanov F, Sugauchi F, Mano S, Maeshiro T, et al. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. *J Virol* 2009;**83**(20):10538–47.
- Ozasa A, Tanaka Y, Orito E, Sugiyama M, Kang JH, Hige S, et al. Influence of genotypes and precore mutations on fulminant or chronic outcome of acute hepatitis B virus infection. *Hepatology* 2006;**44**(2):326–34.
- Matsuura K, Tanaka Y, Hige S, Yamada G, Murawaki Y, Komatsu M, et al. Distribution of hepatitis B virus genotypes among patients with chronic infection in Japan shifting toward an increase of genotype A. *J Clin Microbiol* 2009;**47**(5):1476–83.
- Kao JH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* 2000;**118**(3):554–9.
- Kusakabe A, Tanaka Y, Mochida S, Nakayama N, Inoue K, Sata M, et al. Case-control study for the identification of virological factors associated with fulminant hepatitis B. *Hepatol Res* 2009;**39**(7):648–56.
- Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988;**69**(Pt 10):2575–83.
- Kosaka Y, Takase K, Kojima M, Shimizu M, Inoue K, Yoshida M, et al. Fulminant hepatitis B: induction by hepatitis B virus mutants defective in the precore region and incapable of encoding e antigen. *Gastroenterology* 1991;**100**(4):1087–94.
- Liang TJ, Hasegawa K, Rimon N, Wands JR, Ben-Porath E. A hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *N Engl J Med* 1991;**324**(24):1705–9.
- Omata M, Ehata T, Yokosuka O, Hosoda K, Ohto M. Mutations in the precore region of hepatitis B virus DNA in patients with fulminant and severe hepatitis. *N Engl J Med* 1991;**324**(24):1699–704.
- Imamura T, Yokosuka O, Kurihara T, Kanda T, Fukai K, Imazeki F, et al. Distribution of hepatitis B viral genotypes and mutations in the core promoter and precore regions in acute forms of liver disease in patients from Chiba, Japan. *Gut* 2003;**52**(11):1630–7.
- Inoue J, Ueno Y, Wakui Y, Fukushima K, Kondo Y, Kakazu E, et al. Enhanced replication of hepatitis B virus with frameshift in the precore region found in fulminant hepatitis patients. *J Infect Dis* 2011;**204**(7):1017–25.
- Sato S, Suzuki K, Akahane Y, Akamatsu K, Akiyama K, Yunomura K, et al. Hepatitis B virus strains with mutations in the core promoter in patients with fulminant hepatitis. *Ann Intern Med* 1995;**122**(4):241–8.
- Hasegawa K, Huang J, Rogers SA, Blum HE, Liang TJ. Enhanced replication of a hepatitis B virus mutant associated with an epidemic of fulminant hepatitis. *J Virol* 1994;**68**(3):1651–9.
- Baumert TF, Rogers SA, Hasegawa K, Liang TJ. Two core promoter mutations identified in a hepatitis B virus strain associated with fulminant hepatitis result in enhanced viral replication. *J Clin Invest* 1996;**98**(10):2268–76.
- Sugiyama M, Tanaka Y, Kurbanov F, Nakayama N, Mochida S, Mizokami M. Influences on hepatitis B virus replication by a naturally occurring mutation in the core gene. *Virology* 2007;**365**(2):285–91.
- Parekh S, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, et al. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J Virol* 2003;**77**(12):6601–12.
- Jammeh S, Tavner F, Watson R, Thomas HC, Karayiannis P. Effect of basal core promoter and pre-core mutations on hepatitis B virus replication. *J Gen Virol* 2008;**89**(Pt 4):901–9.
- Trey C, Lipworth L, Chalmers TC, Davidson CS, Gottlieb LS, Popper H, et al. Fulminant hepatic failure. Presumable contribution to halothane. *N Engl J Med* 1968;**279**(15):798–801.
- Inoue J, Ueno Y, Kogure T, Nagasaki F, Kimura O, Obara N, et al. Analysis of the full-length genome of hepatitis B virus in the serum and cerebrospinal fluid of a patient with acute hepatitis B and transverse myelitis. *J Clin Virol* 2008;**41**(4):301–4.
- Shin IT, Tanaka Y, Tateno Y, Mizokami M. Development and public release of a comprehensive hepatitis virus database. *Hepatol Res* 2008;**38**(3):234–43.
- Penna A, Chisari FV, Bertoletti A, Missale G, Fowler P, Giuberti T, et al. Lymphocytes recognize an HLA-A2-restricted epitope within the hepatitis B virus nucleocapsid antigen. *J Exp Med* 1991;**174**(6):1565–70.
- Sugauchi F, Orito E, Ohno T, Tanaka Y, Ozasa A, Kang JH, et al. Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan. *Hepatol Res* 2006;**36**(2):107–14.
- Bunce M, O'Neill CM, Barnardo MC, Krausa P, Browning MJ, Morris PJ, et al. Phototyping: comprehensive DNA typing for HLA-A, B, C, DRB1, DRB3, DRB4, DRB5 & DQB1 by PCR with 144 primer mixes utilizing sequence-specific primers (PCR-SSP). *Tissue Antigens* 1995;**46**(5):355–67.
- Kinouchi Y, Matsumoto K, Negoro K, Takagi S, Takahashi S, Hiwatashi N, et al. HLA-B genotype in Japanese patients with Crohn's disease. *Dis Colon Rectum* 2003;**46**(10 Suppl):S10–4.
- Okamoto H, Yotsumoto S, Akahane Y, Yamanaka T, Miyazaki Y, Sugai Y, et al. Hepatitis B viruses with precore region defects prevail in persistently infected hosts along with seroconversion to the antibody against e antigen. *J Virol* 1990;**64**(3):1298–303.
- Okamoto H, Tsuda F, Akahane Y, Sugai Y, Yoshida M, Moriyama K, et al. Hepatitis B virus with mutations in the core promoter for an e antigen-negative phenotype in carriers with antibody to e antigen. *J Virol* 1994;**68**(12):8102–10.
- Bertoletti A, Sette A, Chisari FV, Penna A, Leverro M, De Carli M, et al. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 1994;**369**(6479):407–10.
- Sendi H, Mehrab-Mohseni M, Shahrz S, Norder H, Alavian SM, Noorinayer B, et al. CTL escape mutations of core protein are more frequent in strains of HBeAg negative patients with low levels of HBV DNA. *J Clin Virol* 2009;**46**(3):259–64.
- Akarca US, Lok AS. Naturally occurring hepatitis B virus core gene mutations. *Hepatology* 1995;**22**(1):50–60.
- Abbott WG, Tsai P, Leung E, Trevarton A, Ofanoa M, Hornell J, et al. Associations between HLA class I alleles and escape mutations in the hepatitis B virus core gene in New Zealand-resident Tongans. *J Virol* 2010;**84**(1):621–9.

