comparison among HBV genotypes/subgenotypes free of confounding variables such as host and environmental factors. Furthermore, the lack of immune responses in chimeric mice enables analysis of sheer virological differences. These experimental models may contribute to understanding the influence of genotypes/subgenotypes on clinical outcomes of HBV infection and response to various antiviral treatments.

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References

1. Lee WM. Hepatitis B virus infection. N Engl J Med 1997;337:1733-1745.
2. 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: 2575-2583.
3. Arauz-Ruiz P, Norder H, Robertson BH, Magnius LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059-2073.
4. Norder H, Courouce AM, Magnius LO. Complete genomes, phylogenetic relatedness, and structural proteins of six strains of the hepatitis B virus, four of which represent two new genotypes. Virology 1994;198:489-503.
5. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67-74.
6. Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus–large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285-1293.
7. Magnius LO, Norder H. Subtypes, genotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology 1995;38:24-34.
8. Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329-338.
9. Bowyer SM, van Staden L, Kew MC, Sim JG. A unique segment of the hepatitis B virus group A genotype identified in isolates from South Africa. J Gen Virol 1997;78:1719-1729.
10. Kramvis A, Weitzmann L, Owiredu WK, Kew MC. Analysis of the complete genome of subgroup A' hepatitis B virus isolates from South Africa. J Gen Virol 2002;83:835-839.
11. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M, et al. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811-820.
12. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, et al. Hepatitis B virus of genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985-5992.
13. Sugauchi F, Orito E, Ichida T, Kato H, Sakugawa H, Kakumu S, Ishida T, et al. Epidemiologic and virologic characteristics of hepatitis B virus genotype B having the recombination with genotype C. Gastroenterology 2003;124:925-932.
14. Kramvis A, Kew M, Francois G. Hepatitis B virus genotypes. Vaccine 2005;23:2409-2423.
15. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. Intervirology 2004; 47:289-309.
16. Kramvis A, Kew MC. Relationship of genotypes of hepatitis B virus to mutations, disease progression and response to antiviral therapy. J Viral Hepat 2005;12:456-464.
17. Liu CJ, Kao JH, Chen DS. Therapeutic implications of hepatitis B virus genotypes. Liver Int 2005;25:1097-1107.
18. Schaefer S. Hepatitis B virus: significance of genotypes. J Viral Hepat 2005;12:111-124.
19. Erhardt A, Blondin D, Hauck K, Sagir A, Kohnle T, Heintges T, et al. Response to interferon alfa is hepatitis B virus genotype dependent: genotype A is more sensitive to interferon than genotype D. Gut 2005;54:1009-1013.
20. Orito E, Mizokami M, Sakugawa H, Michitaka K, Ishikawa K, Ichida T, 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;33:218-223.
21. Thakur V, Guptan RC, Kazim SN, Malhotra V, Sarin SK. Profile, spectrum and significance of HBV genotypes in chronic liver disease patients in the Indian subcontinent. J Gastroenterol Hepatol 2002;17:165-170.
22. Janssen HL, van Zonneveld M, Senturk H, Zeuzem S, Akarca US, Cakaloglu Y, et al. Pegylated interferon alfa-2b alone or in combination with lamivudine for HBeAg-positive chronic hepatitis B: a randomised trial. Lancet 2005;365:123-129.
23. Westland C, Delaney Wt, Yang H, Chen SS, Marcellin P, Hadziyannis S, et al. Hepatitis B virus genotypes and virologic response in 694 patients in phase III studies of adefovir dipivoxil. Gastroenterology 2003;125:107-116.
24. Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. Proc Natl Acad Sci U S A 1987;84:1005-1009.
25. Tsurimoto T, Fujiyama A, Matsubara K. Stable expression and replication of hepatitis B virus genome in an integrated state in a human hepatoma cell line transfected with the cloned viral DNA. Proc Natl Acad Sci U S A 1987;84:444-448.
26. Gerber MA, Sells MA, Chen ML, Thung SN, Tabibzadeh SS, Hood A, et al. Morphologic, immunohistochemical, and ultrastructural studies of the production of hepatitis B virus in vitro. Lab Invest 1988;59:173-180.
27. Acs G, Sells MA, Purcell RH, Price P, Engle R, Shapiro M, et al. Hepatitis B virus produced by transfected Hep G2 cells causes hepatitis in chimpanzees. Proc Natl Acad Sci U S A 1987;84:4641-4644.
28. Tacke F, Gehrke C, Luedde T, Heim A, Manns MP, Trautwein C. Basal core promoter and precore mutations in the hepatitis B virus genome enhance replication efficacy of Lamivudine-resistant mutants. J Virol 2004;78:8524-8535.
29. Heckel JL, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. Cell 1990;62:447-456.
30. Rhim JA, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Replacement of diseased mouse liver by hepatic cell transplantation. Science 1994; 263:1149-1152.
31. Meuleman P, Libbrecht L, De Vos R, de Hemptinne B, Gevaert K, Vandekerckhove J, et al. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. HEPATOLOGY 2005;41: 847-856.
32. Dandri M, Burda MR, Torok E, Pollok JM, Iwanska A, Sommer G, et al. Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. HEPATOLOGY 2001;33:981-988.
33. Meuleman P, Libbrecht L, Wieland S, De Vos R, Habib N, Kramvis A, et al. Immune suppression uncovers endogenous cytopathic effects of the hepatitis B virus. J Virol 2006;80:2797-2807.
34. Tsuge M, Hiraga N, Takaishi H, Noguchi C, Oga H, Imamura M, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. HEPATOLOGY 2005;42:1046-1054.
35. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927-933.

36. Kimura T, Ohno N, Terada N, Rokuhara A, Matsumoto A, Yagi S, et al. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. J Biol Chem 2005;280:21713-21719.
37. Fujiwara K, Tanaka Y, Paulon E, Orito E, Sugiyama M, Ito K, et al. Novel type of hepatitis B virus mutation: replacement mutation involving a hepatocyte nuclear factor 1 binding site tandem repeat in chronic hepatitis B virus genotype E. J Virol 2005;79:14404-14410.
38. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901-912.
39. Tanaka Y, Hasegawa I, Kato T, Orito E, Hirashima N, Acharya SK, et al. A case-control study for differences among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. HEPATOLOGY 2004;40:747-755.
40. Foti DM, Welihinda A, Kaufman RJ, Lee AS. Conservation and divergence of the yeast and mammalian unfolded protein response. Activation of specific mammalian endoplasmic reticulum stress element of the grp78/BiP promoter by yeast Hac1. J Biol Chem 1999;274:30402-30409.
41. Lee AS. The glucose-regulated proteins: stress induction and clinical applications. Trends Biochem Sci 2001;26:504-510.
42. Chan HL, Tsang SW, Liew CT, Tse CH, Wong ML, Ching JY, et al. Viral genotype and hepatitis B virus DNA levels are correlated with histological liver damage in HBeAg-negative chronic hepatitis B virus infection. Am J Gastroenterol 2002;97:406-412.
43. Akuta N, Suzuki F, Kobayashi M, Tsubota A, Suzuki Y, Hosaka T, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315-321.
44. 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;44:326-334.
45. Zhang X, Zoulim F, Habersetzer F, Xiong S, Trepo C. Analysis of hepatitis B virus genotypes and pre-core region variability during interferon treatment of HBe antigen negative chronic hepatitis B. J Med Virol 1996;48:8-16.
46. Heijtink RA, Paulij W, van Roosmalen M, Hellings JA, Niesters HG, Schalm SW, et al. Characteristics of the early phase of chronicity in acute hepatitis B infection. J Med Virol 1999;57:331-336.
47. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, et al. Viral genotypes and response to interferon in patients with acute prolonged hepatitis B virus infection of adulthood in Japan. J Med Virol 2002;68:522-528.
48. Lindh M, Horal P, Norkrans G. Acute hepatitis B in western Sweden–genotypes and transmission routes. Infection 2000;28:161-163.
49. Kew MC, Kramvis A, Yu MC, Arakawa K, Hodkinson J. Increased hepatocarcinogenic potential of hepatitis B virus genotype A in Bantu-speaking sub-saharan Africans. J Med Virol 2005;75:513-521.
50. Orito E, Mizokami M, Ina Y, Moriyama EN, Kameshima N, Yamamoto M, et al. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. Proc Natl Acad Sci U S A 1989;86:7059-7062.

MAJOR ARTICLE

T1653 Mutation in the Box α Increases the Risk of Hepatocellular Carcinoma in Patients with Chronic Hepatitis B Virus Genotype C Infection

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Background. Most patients with chronic hepatitis B virus infection become carriers of inactive virus after hepatitis B e antigen seroconversion; however, a subgroup of patients have persistent abnormal transaminase levels and develop hepatocellular carcinoma after seroconversion.

Methods. In an age-matched case-control study, 40 carriers of inactive virus (mean age ± standard deviation [SD], 50.9 ± 11.1 years), 40 patients with chronic hepatitis (mean age ± SD, 50.2 ± 8.9 years), and 40 patients with hepatocellular carcinoma (mean age ± SD, 50.7 ± 9.4 years) who were infected with hepatitis B virus genotype C and had test results positive for antibody to hepatitis B e antigen were analyzed.

Results. The prevalence of T1653 in the box α was significantly higher among patients with hepatocellular carcinoma than among carriers of inactive virus who did not have hepatocellular carcinoma (70% vs. 25%; $P < .0001$) or chronic hepatitis (70% vs. 35%; $P = .003$). Mutations in the basic core promoter region (T1762/A1764) were frequently found in all groups, regardless of clinical status (in 77.5% of carriers of inactive virus, 77.5% of patients with chronic hepatitis, and 90% of patients with hepatocellular carcinoma). In the multivariate analysis, the presence of T1653, an alanine aminotransferase level of ≥37 U/L, and a platelet count of $<18 \times 10^4$ platelets/mm^3 were independent predictive values for hepatocellular carcinoma (odds ratio [95% confidence interval], 5.05 [1.56–16.35], 12.56 [3.05–51.77], and 11.5 [3.47–38.21], respectively). High α-fetoprotein level was the only independent predictive value for T1653 in patients with hepatocellular carcinoma (odds ratio, 12.67; 95% confidence interval, 1.19–134.17]). Among patients with test results positive for antibody to hepatitis B e antigen who had hepatocellular carcinoma and were infected with different genotypes of hepatitis B virus, the prevalence of T1653 was 40%, 15%, 25%, 25%, 67%, and 23% in patients infected with hepatitis B virus genotypes Aa, Ae, Ba, Bj, C, and D, respectively ($P < .05$ for genotype C vs. genotypes Ae, Ba, Bj, or D).

Conclusions. Our data indicate that the addition of T1653 mutation in the box α to the basic core promoter mutation increases the risk of hepatocellular carcinoma in patients with hepatitis B virus genotype C.

Hepatocellular carcinoma (HCC) is the fifth most frequent cancer and the third leading cause of cancer-related death in the world, with an estimated prevalence of >500,000 cases worldwide per year [1]. It is now accepted that hepatitis B virus (HBV) has a carcinogenic potential in humans. Several mutations in the HBV genome have been reported to occur during the course of persistent viral infection, and there has been increasing evidence of an association between these molecular alterations and the development of HCC in patients with HBV infection.

During persistent HBV infection, carriers frequently undergo seroconversion from hepatitis B e antigen (HBeAg) to the corresponding antibody (anti-HBe). Most patients who acquire chronic HBV infection with HBV genotype C (which is a common genotype in East

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Asian countries) by perinatal transmission become carriers of inactive virus after seroconversion. A subgroup of patients have persistent abnormal serum transaminase levels and develop HCC in the anti-HBe–positive phase. Many of these patients have active viral replication and are infected with several mutant viruses. The association between different clinical events after seroconversion and specific HBV genomic mutations has not been clearly defined.

Mutations in the basic core promoter (BCP) region at nucleotides (nt) 1762/1764 (T1762/A1764) and mutation in the precore (preC) region at nt 1896 (A1896) are associated with seroconversion and persistent viral replication. It is noteworthy that both BCP and preC mutations are often found in patients with advanced liver disease, (e.g., HCC) [2–8]. The T1762/A1764 mutation alters HBeAg production at the transcription level, and the A1896 in the preC region terminates translation of the precursor protein, abrogates HBeAg production, and results in seroconversion. A1896 was also reported previously to be associated with severe forms of chronic liver disease [7,8].

HBV has been classified into 8 major genotypes with use of the complete nucleotide sequence of the viral genome [10]. HBV genotypes not only have distinct geographical distributions [7, 11, 12] but also have different clinical manifestations and responses to therapy (e.g., IFN therapy). Furthermore, HBeAg positivity and levels of HBV DNA, which are controlled by specific mutations, differ between HBV genotypes (e.g., the BCP double mutation is more prevalent among strains of HBV genotype C, followed by HBV genotype A, and the A1896 mutation is frequently found in HBV genotypes B and D) [13–16].

There have been many studies involving viral mutations associated with clinical features, but most previous studies have ignored age, sex, HBeAg status, and HBV genotypes. In Japan, most patients with HCC experience seroconversion (i.e., they are anti-HBe positive) and have HBV genotype C; therefore, we performed an age-matched case-control study among anti-HBe–positive patients infected with HBV genotype C (including carriers of inactive virus, patients with chronic hepatitis, and patients with HCC) to determine the specific HBV genome mutations associated with disease progression.

PATIENTS AND METHODS

Serum samples. Serum samples were obtained from 211 patients from different regional areas worldwide. A total of 120 patients from Japan who were infected with HBV genotype C (40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC) were matched with control subjects according to age and HBe status. Control serum samples were obtained from patients with HCC who were positive for anti-HBe and who were infected with HBV genotype Aa (10 subjects), Ae (13), Ba (20), Bj (20), C (15), and D (13). Control subjects were from Hong Kong (19 subjects), Japan (36), and the United States (36). The majority of patients infected with HBV genotypes Aa, Ba, Bj, and C were Asian, and the majority of patients infected with HBV genotypes Ae and D were white and black. None of the subjects had serological test results positive for markers of infection with hepatitis C virus or HIV-1.

The study protocol was approved by ethics committees of the participating institutions in accordance with the 1975 Helsinki declaration. Informed consent was obtained from each patient.

Serological assays for HBV markers. HBeAg and anti-HBe were detected by chemiluminescent EIA (Lumipulse *f*, Fujirebio). HBV genotypes were determined by the restriction fragment–length polymorphism method on the S gene sequence amplified by PCR [29] and ELISA with monoclonal antibodies directed to distinct epitopes on the preS2 region products [18], with use of commercial kits (HBV genotype EIA; Institute of Immunology). The genotypes were also confirmed with use of a phylogenetic tree analysis. α-Fetoprotein and serum protein induced by the absence of vitamin K (antagonist II) were examined with use of chemiluminescent EIA.

Amplification and sequencing of the core promoter and the precore region plus core gene. HBV DNA sequences bearing the core promoter and preC or core regions were amplified by PCR with heminested primers by the method described elsewhere [19]. Thereafter, PCR products were sequenced directly with Prism Big Dye (Applied Biosystems) in the ABI 3100 DNA automated sequencer (Applied Biosystems). Accession numbers for all strains are AB236515–AB236634.

Case-control study. A carrier of inactive virus was defined as an HBsAg-positive individual with normal alanine aminotransferase (ALT) levels for a 2-year period (with at least 4 evaluations at 3-month intervals) and without the presence of portal hypertension. Chronic hepatitis was defined as persistent elevation of ALT levels (> 1.5 × upper limit of normal [35 U/L]) during a 6-month period (with at least 3 evaluations at 2-month intervals) without a decrease in platelet count or albumin level, and hypersplenism (splenomegaly on ultrasonographic examination). Twenty-one patients were confirmed to have chronic hepatitis by means of a fine-needle biopsy of the liver. Staging and grading (expressed as mean value ± SD [95% CI]) were 1.24 ± 0.64 (0.99–1.58) and 1.36 ± 0.58 (1.07–1.59), respectively, as previously described [30]. None had received antiviral treatment during the follow-up period. Of 40 patients with HCC, 23 patients received a diagnosis of HCC on the basis of a pathologic examination, and 17 patients received a diagnosis of HCC on the basis of results of abdominal ultrasonography, angiography, CT, or MRI, as well as an elevated serum α-fetoprotein level (≥400 ng/mL).

Statistical evaluation. Data were expressed as mean ±

SD. Statistical analyses were performed using χ^2 test and Fisher's exact test for categorical variables. Mann-Whitney U test or 1-way analysis of variance were used for continuous variables, as appropriate. Mantel-Haenszel χ^2 test was used to analyze the trend of frequencies of viral mutations. Multivariate analyses with logistic regression were used to determine the independent factors associated with HCC and T1653. Differences were considered to be significant for P values <.05. The statistical analysis software used was Stata software, version 8.0 (StataCorp).

RESULTS

Table 1 compares ALT level, platelet count, and HBV DNA level, as well as mutations in the box α (enhancer II), core promoter, and preC region, among 40 carriers of inactive virus, 40 patients with chronic hepatitis, and 40 patients with HCC who were infected with HBV genotype C in an age-matched case-control study. ALT and HBV DNA levels were significantly lower among carriers of inactive virus than among patients with chronic hepatitis or patients with HCC ($P<.0001$ and $P = .001$, respectively). Platelet count was lower among patients with HCC than among carriers of inactive virus or patients with chronic hepatitis ($P<.0001$).

The frequency of the T1653 mutation in the box α was significantly higher among patients with HCC (70%) than among carriers of inactive virus (25%) or patients with chronic hepatitis (35%; $P<.0001$) (table 1). Of interest, the T1653 mutation had an opposite correlation with the M1753 mutation. The prevalence of T1762/A1764 was high in all clinical status groups, with no statistically significant difference between groups (table 1). The trend of the frequency of T1653, increasing from carriers of inactive virus to patients with chronic hepatitis to patients with HCC, was analyzed by Mantel-Haenszel χ^2 test (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$) (figure 1). The trend of the frequency of T1762/A1764 was not statistically significant ($P = .1502$) (figure 1).

The attributable risk of multiple factors, including sex, HBV DNA level, ALT level, platelet count, and the presence of the T1653, M1753, T1762/A1764, and A1896 mutations for HCC in the HBV carriers was determined by multiple logistic regression analysis (table 2). There was a statistically significant association between development of HCC and ALT level >37 U/L (OR, 12.56; 95% CI, 0.55–6.21; $P<.0001$) and platelet count <18 × 10^4 platelets/mm^3 (OR, 11.5; 95% CI, 3.47–38.21; $P<.0001$). The T1653 mutation was still significantly associated with the development of HCC (OR, 5.05; 95% CI, 1.56–16.35; $P = .007$).

The attributable risk of multiple factors, including HBV DNA level, ALT level, platelet count, α-fetoprotein level, protein in-

Table 1. Demographic, clinical, and virologic characteristics of patients infected with hepatitis B virus (HBV) genotype C who were matched for age and hepatitis B e antigen (HBeAg) status.

Variable	Clinical status			
	Carriage of inactive virus (n = 40)	Chronic hepatitis (n = 40)	Hepatocellular carcinoma (n = 40)	P
Male sex	31 (77.5)	37 (92.5)	36 (90)	.171
Age, years	50.9 ± 11.1	50.2 ± 8.9	50.7 ± 9.4	Matched
HBeAg positive	0 (0)	0 (0)	0 (0)	Matched
Anti-HBeAg positive	40 (100)	40 (100)	40 (100)	Matched
HBV genotype C	40 (100)	40 (100)	40 (100)	Matched
Alanine transaminase level, U/L[a]	20.8 ± 7.6	102 ± 108.7	83.2 ± 84.8	.0001
Platelet count, ×10^4 platelets/mm^3[b]	20.7 ± 3.1	17.4 ± 4.1	12.8 ± 5.7	.0001
HBV DNA level, LGE/mL[c]	4.3 ± 0.8	5.9 ± 1.5	5.4 ± 1.5	<.0001
Mutation in the box α: T1653[d]	10 (25)	14 (35)	28 (70)	<.0001
Mutation in the core promoter				
M1753	10 (25)	6 (15)	9 (22.5)	.609
T1762/A1764	31 (77.5)	31 (77.5)	36 (90)	.289
Mutation in the precore region: A1896	25 (62.5)	26 (65)	25 (62.5)	1.0

NOTE. Data are no. (%) of patients or mean value ± SD. Anti-HBeAg, antibody to HBeAg; LGE, log genome equivalents.

[a] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[b] $P<.0001$ for patients with hepatocellular carcinoma vs. carriers of inactive virus or patients with chronic hepatitis; $P = .002$ for carriers of inactive virus vs. patients with chronic hepatitis.

[c] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

[d] $P<.0001$ for carriers of inactive virus vs. patients with chronic hepatitis; $P = .001$ for carriers of inactive virus vs. patients with hepatocellular carcinoma.

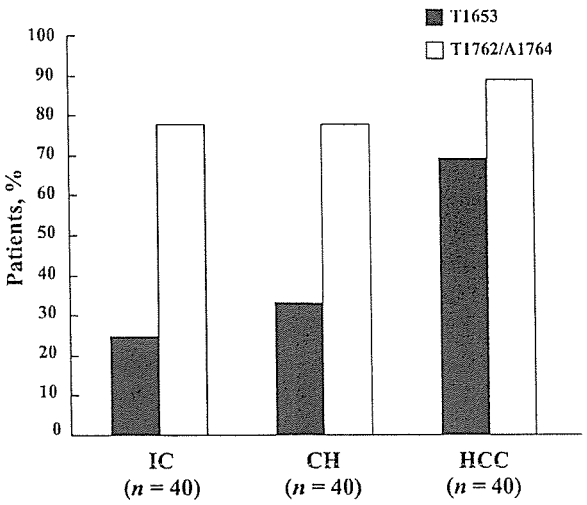

Figure 1. Prevalence of T1653 box α and T1762/A1764 basic core promoter mutations among patients with chronic hepatitis B virus infection, stratified by clinical status. The trend of the frequency of the T1653 mutation was analyzed by Mantel-Haenszel χ^2 test. The OR estimate is an approximation of the OR for carriers of inactive virus (IC), patients with chronic hepatitis (CH), and patients with hepatocellular carcinoma (HCC) having a strain with the mutation (OR, 2.48; 95% CI, 1.59–3.85; $P = .0001$). The trend of the frequency of the T1762/A1764 mutation was not statistically significant according to the Mantel-Haenszel χ^2 test ($P = .1502$).

duced by the absence of vitamin K (antagonist II) level, for T1653 in patients with HCC with HBV genotype C infection was determined by multiple logistic regression analysis (table 3). An α-fetoprotein level >300 ng/mL was the only independent predictive value for the presence of the T1653 mutation in patients with HCC with HBV genotype C infection (OR, 12.67; 95% CI, 1.19–134.17; $P = .035$).

Table 4 compares sex, age, and mutations in the box α, core promoter, and preC region among patients infected with HBV genotypes Aa (10 patients), Ae (13), Ba (20), Bj (20), C (15), and D (13) with the same variables among patients with HCC. Mean age was significantly higher among patients with HBV genotype Bj infection, compared with patients with HBV genotype Ba, genotype C, and genotype D infection ($P < .05$). The prevalence of T1653 among patients with HBV genotype C infection (66.7%) was significantly higher than it was among patients infected with other genotypes (15%–25%; $P < .05$), excluding patients infected with HBV genotype Aa. The prevalence of T1762/A1764 among patients with HBV genotype Ba infection (85%) and HBV genotype C infection (86.7%) was also significantly higher than it was among patients infected with other genotypes (20%–50%; $P < .05$). The prevalence of A1896 among patients with HBV genotype Aa infection and HBV genotype Ae infection was significantly lower than it was among patients infected with other genotypes ($P < .05$).

DISCUSSION

Many previous studies have reported that the clinical course of chronic HBV infection may be modified by several specific viral mutations [5, 20, 21], although the significance of such specific mutations in patients with chronic hepatitis B remains controversial. Because most studies have not controlled for different variables, such as age, HBV genotype, and HBe status, it is unknown whether the mutations were associated with disease progression, greater age of the patient, the specific HBV genotype or subtype, or HBe status. In this study, to exclude any biases, we performed an age-matched case-control study involving only anti-HBe–positive patients infected with HBV genotype C.

In the present case-control study, the prevalence of T1653 was found to be significantly higher among patients with HCC, compared with carriers of inactive virus and patients with chronic hepatitis with HBV genotype C infection; however, the prevalence of T1762/A1764 was high in all clinical status groups. During the anti-HBe–positive phase of infection, T1653 was more reliable than T1762/A1764 as a predicting factor for

Table 2. Multivariate analysis of variables with independent predictive value for development of hepatocellular carcinoma among a group of 120 patients with hepatitis B virus infection.

Variable	OR (95% CI)	*P*
Sex		
Female	1	
Male	5.06 (0.85–30.15)	.075
HBV DNA level		
<4.8 LGE/mL	1	
≥4.8 LGE/mL	0.34 (0.09–1.21)	.096
Alanine transaminase level		
<37 U/L	1	
≥37 U/L	12.56 (3.05–51.77)	.0001[a]
Platelet count		
≥18 × 10^4 platelets/mm^3	1	
<18 × 10^4 platelets/mm^3	11.51 (3.47–38.21)	.0001[a]
T1653 mutation		
No	1	
Yes	5.05 (1.56–16.35)	.007[a]
M1753 mutation		
No	1	
Yes	1.23 (0.31–5.04)	.770
T1762/A1764 mutation		
No	1	
Yes	2.67 (0.57–12.54)	.214
A1896 mutation		
No	1	
Yes	0.96 (0.29–3.11)	.943

NOTE. Each OR was adjusted for age and other variables in the analysis. LGE, log genome equivalents.

[a] Statistically significant.

Table 3. Multivariate analysis of variables with independent predictive value for the presence of the T1653 mutation among 40 patients with hepatocellular carcinoma.

Variable	OR (95% CI)	*P*
HBV DNA level		
<4.9 LGE/mL	1	
≥4.9 LGE/mL	0.89 (0.16–4.79)	.899
ALT level		
<53 U/L	1	
≥53 U/L	1.72 (0.29–9.96)	.541
Platelet count		
≥12 × 10^4 platelets/mm^3	1	
<12 × 10^4 platelets/mm^3	1.39 (0.28–7.02)	.683
α-Fetoprotein level		
<300 ng/mL	1	
≥300 ng/mL	12.67 (1.19–134.17)	.035[a]
PIVKA-2 level		
<50 mAU/mL	1	
≥50 mAU/mL	0.25 (0.05–1.43)	.120

NOTE. Each OR was adjusted for age and other variables in the table. PIVKA-2, protein induced by the absence of vitamin K (antagonist II).

[a] Statistically significant.

the development of HCC. In fact, in the multivariate analysis, the presence of T1762/A1764 was not an independent predictor of HCC, but ALT level >37 U/L, platelet count <18 × 10^4 platelets/mm^3, and the presence of T1653 were independent predictors of HCC. The T1653 mutation had also been reported by Takahashi et al. [17]; they reported that this specific mutation was prevalent among Japanese patients with HCC, although their study was not a case-control study. These results do not deny that T1762/A1764 is associated with hepatocarcinogenesis, because poor prognosis associated with HBV genotype C infection, compared to that associated with HBV genotype B (Ba and Bj) infection, correlated with a high prevalence of T1762/A1764 [2, 9, 16], indicating that the BCP double mutation is associated with a high potential for hepatocarcinogenesis. The appearance of the T1653 mutation after the occurrence of the T1762/A1764 mutation (the T1762/A1764 mutation usually occurs earlier than the T1653 mutation) could indicate that the virulence of HBV is increasing, which could result in the development of HCC. In the multivariate analysis, however, HBV DNA level was no longer a predicting factor for HCC. One of the reasons for this is that the HBV DNA data used in this study were obtained at the time of diagnosis of HCC. A recent prospective study from Taiwan has indicated that high HBV DNA levels at baseline and infection with HBC genotype C were independent predictors for HCC, but the mean viral load at the time of diagnosis of HCC was significantly lower than at baseline [27]. Although our data could not indicate an association between HBV DNA level and hepatocarcinogenesis, if we could measure the HBV DNA level before diagnosis of HCC, it might found to be be a predicting factor for HCC. Furthermore, an examination of the characteristics of patients with HCC who had the T1653 mutation showed that an elevated α-fetoprotein level (≥300 ng/mL) was the only predictor for the development of HCC in patients with the T1653 mutation. It has been reported that α-fetoprotein level is useful not only for diagnosis but also as a prognostic indicator for patients with HCC [22, 23], suggesting that the T1653 mutation might be associated with poor prognosis for patients with HCC.

The prevalence of several mutations among patients with HCC differed from that among patients with different HBV genotypes (Aa, Ae, Ba, Bj, C, and D) (table 4). The prevalence

Table 4. Demographic and virological characteristics of patients with hepatocellular carcinoma who were positive for antibody to hepatitis B e antigen (anti-HBe), by hepatitis B virus (HBV) genotype.

	HBV genotype						
Variable	Aa (*n* = 10)	Ae (*n* = 13)	Ba (*n* = 20)	Bj (*n* = 20)	C (*n* = 15)	D (*n* = 13)	*P*
Male	10 (100)	12 (92.3)	18 (90)	15 (75)	15 (100)	13 (100)	.10
Age, years[a]	54.4 ± 7.7	55.3 ± 4.4	54.4 ± 14.8	64.9 ± 9.6	47.9 ± 7.6	53.5 ± 8.3	.0002
HBeAg positive	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	Matched
Anti-HBe positive	10 (100)	13 (100)	20 (100)	20 (100)	15 (100)	13 (100)	Matched
Mutation in the box α: T1653[b]	4 (40)	2 (15.4)	5 (25)	5 (25)	10 (66.7)	3 (23.1)	.039
Mutations in the core promoter region							
M1753	3 (30)	3 (23.1)	5 (25)	4 (20)	2 (13.3)	1 (7.7)	.759
T1762/A1764[c]	5 (50)	6 (46.2)	17 (85)	4 (20)	13 (86.7)	5 (38.5)	<.0001
Mutation in the precore region: A1896[d]	0 (0)	0/13 (0)	9/20 (45)	15/20 (75)	9/15 (60)	8/13 (61.5)	<.0001

NOTE. Data are no. (%) of patients or mean value ± SD. HBeAg, hepatitis B e antigen.

[a] $P < .05$ for Bj vs. Ba or D; $P < .0001$ for Bj vs. C.

[b] $P < .05$ for C vs. Ba or Bj or D; $P < .01$ for Ae vs. C.

[c] $P < .05$ for Ae vs. Ba or C; $P < .01$ for D vs. Ba or C; $P < .0001$ for Bj vs Ba or C.

[d] $P < .05$ for Ba vs. Aa or Ae; $P < .005$ for Aa vs. C or D and for Ae vs. Ba or C or D; $P < .0001$ for Bj vs Aa or Ae.

of T1653 was the highest among patients with HBV genotype C infection, followed by those with HBV genotype Aa infection, although the number of patient with HBV genotype Aa infection was too small for any conclusions to be drawn. The prevalence of T1762/A1764 was higher among patients with HBV genotype Ba and HBV genotype C infection than among patients infected with other genotypes. HBV genotype Ba has a sequence that closely resembles that of HBV genotype C in the core promoter region, because it is recombinant HBV between HBV genotype Bj and HBV genotype C from nucleotides 1740 to 2485. Although A1896 was not found in HBV genotype Aa and HBV genotype Ae, as has been reported elsewhere [15], HBV genotype Aa had some specific mutations upstream of the preC initiation codon and encapsidation signal site. Therefore, several HBV genotype–specific mutations would be associated with different mechanisms on seroconversion or HBV replication for each genotype or subtype.

Buckwold et al. [24] reported that T1762/A1764 can no longer bind liver-enriched transcription factors and that the transcription of precore RNA and the expression of HBeAg were reduced. Thereafter, Li et al. [25] reported that this mutation not only removed the nuclear receptor–binding site but also created a hepatic nuclear factor 1 transcription factor–binding site. As for a factor correlated with BCP, the core upstream regulatory sequence, which has a strong stimulation effect on the BCP, was reported. In an earlier article by Yu et al. [28], the box α elements (nucleotides 1646–1668) individually stimulated promoter activity >100-fold. The T1653 mutation converts the box α binding site for CCAAT/enhancer-binding protein and related factors into the perfect palindromic sequence 1648-TCTTATATAAGA, which might enhance binding affinity and core promoter/enhancer II activity. Therefore, it is possible that the mutation in the box α influenced the HBe production and viral replication through the BCP activity. In addition, the T1653 mutation corresponds to an amino acid change from histidine to tyrosine at aa 94 of the X protein, so this alteration of X protein might be hepatocarcionogenesis. Gunther et al. [26] analyzed T1653, T1762, and A1764 mutations in the context of an in vitro study involving wild-type HBV (genotype D, AF043594), and they reported that the preC mRNA and HBeAg secretion was reduced, but the amount of progeny virus DNA in the cells and in the culture medium increased only marginally (if at all), as determined by Southern blot analysis. However, because the genotype was different from that in our study (genotype D vs. genotype C) and the mutant type included not only T1653, T1762, and A1764 mutations but also other mutations in the core promoter, it is possible that some other mutation influenced the results in the earlier study.

In conclusion, the addition of the T1653 mutation in the box α to the BCP mutation increases the risk of HCC in patients with HBV genotype C infection, suggesting that HBV with both the T1653 mutation and the BCP double mutation in patients with chronic hepatitis B should be eradicated by antiviral therapy. Functional analyses of HBV strains with the T1653 mutation are needed in vitro and in vivo.

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References

1. Parkin DM. International variation. Oncogene **2004**; 23:6329–40.
2. Kao JH, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. Gastroenterology **2003**; 124:327–34.
3. Baptista M, Kramvis A, Kew MC. High prevalence of 1762(T) 1764(A) mutations in the basic core promoter of hepatitis B virus isolated from black Africans with hepatocellular carcinoma compared with asymptomatic carriers. Hepatology **1999**; 29:946–53.
4. Blackberg J, Kidd-Ljunggren K. Mutations within the hepatitis B virus genome among chronic hepatitis B patients with hepatocellular carcinoma. J Med Virol **2003**; 71:18–23.
5. Lindh M, Gustavson C, Mardberg K, Norkrans G, Dhillon AP, Horal P. Mutation of nucleotide 1,762 in the core promoter region during hepatitis B e seroconversion and its relation to liver damage in hepatitis B e antigen carriers. J Med Virol **1998**; 55:185–90.
6. Laskus T, Rakela J, Nowicki MJ, Persing DH. Hepatitis B virus core promoter sequence analysis in fulminant and chronic hepatitis B. Gastroenterology **1995**; 109:1618–23.
7. Kobayashi M, Arase Y, Ikeda K, et al. Precore wild-type hepatitis B virus with G1896 in the resolution of persistent hepatitis B virus infection. Intervirology **2003**; 46:157–63.
8. Raimondo G, Schneider R, Stemler M, Smedile V, Rodino G, Will H. A new hepatitis B virus variant in a chronic carrier with multiple episodes of viral reactivation and acute hepatitis. Virology **1990**; 179: 64–8.
9. 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**; 124:925–32.
10. Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol **1988**; 69:2575–83.
11. Magnius LO, Norder H. Subtypes, genotypes, and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology **1995**; 38:24–34.
12. Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology **2001**; 34:590–4.
13. Lindh M, Horal P, Dhillon AP, Norkrans G. Hepatitis B virus DNA levels, precore mutations, genotypes and histological activity in chronic hepatitis. J Viral Hepat **2000**; 7:258–67.
14. Lindh M, Hannoun C, Dhillon AP, Norkrans G, Horal P. Core promoter mutations and genotypes in relation to viral replication and liver damage in East Asian hepatitis B virus carriers. J Infect Dis **1999**; 179: 775–82.
15. Tanaka Y, Hasegawa I, Kato T, et al. A case-control study for differences

among hepatitis B virus infections of genotypes A (subtypes Aa and Ae) and D. Hepatology **2004**; 40:747–55.
16. 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**; 33:218–23.
17. Takahashi K, Akahane Y, Hino K, Ohta Y, Mishiro S. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. Arch Virol **1998**; 143:2313–26.
18. Usuda S, Okamoto H, Tanaka T, et al. Differentiation of hepatitis B virus genotypes D and E by ELISA using monoclonal antibodies to epitopes on the preS2-region product. J Virol Methods **2000**; 87:81–9.
19. Sugauchi F, Mizokami M, Orito E, et al. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J Gen Virol **2001**; 82:883–92.
20. Chu CM, Yeh CT, Lee CS, Sheen IS, Liaw YF. Precore stop mutant in HBeAg-positive patients with chronic hepatitis B: clinical characteristics and correlation with the course of HBeAg-to-anti-HBe seroconversion. J Clin Microbiol **2002**; 40:16–21.
21. Huy TT, Ushijima H, Quang VX, et al. Characteristics of core promoter and precore stop codon mutants of hepatitis B virus in Vietnam. J Med Virol **2004**; 74:228–36.
22. Tangkijvanich P, Anukulkarnkusol N, Suwangool P, et al. Clinical characteristics and prognosis of hepatocellular carcinoma: analysis based on serum alpha-fetoprotein levels. J Clin Gastroenterol **2000**; 31:302–8.
23. Izumi R, Shimizu K, Kiriyama M, et al. Alpha-fetoprotein production by hepatocellular carcinoma is prognostic of poor patient survival. J Surg Oncol **1992**; 49:151–5.
24. Buckwold VE, Xu Z, Chen M, Yen TS, Ou JH. Effects of a naturally occurring mutation in the hepatitis B virus basal core promoter on precore gene expression and viral replication. J Virol **1996**; 70:5845–51.
25. Li J, Buckwold VE, Hon MW, Ou JH. Mechanism of suppression of hepatitis B virus precore RNA transcription by a frequent double mutation. J Virol **1999**; 73:1239–44.
26. Gunther S, Piwon N, Will H. Wild-type levels of pregenomic RNA and replication but reduced pre-C RNA and e-antigen synthesis of hepatitis B virus with C(1653) → T, A(1762) → T and G(1764) → A mutations in the core promoter. J Gen Virol **1998**; 79:375–80.
27. Yu MW, Yeh SH, Chen PJ, et al. Hepatitis B virus genotype and DNA level and hapatocellular carcinoma: a prospective study in men. J Natl Cancer Inst **2005**; 97:265–72.
28. Yuh CH, Chang YL, Ting LP. Transcriptional regulation of precore and pregenomic RNAs of hepatitis B virus. J Virol **1992**; 66:4073–84.
29. Mizokami M, Nakano T, Orito E, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett **1999**; 450:66–71.
30. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. Hepatology **1994**; 19:1513–20.

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A case–control study of response to lamivudine therapy for 2 years in Japanese and Chinese patients chronically infected with hepatitis B virus of genotypes Bj, Ba and C

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Abstract

Background/aims: In eastern Asian countries, hepatitis B virus (HBV) genotype Ba (HBV/Ba), HBV/Bj and HBV/C are prevalent. The aim was to investigate the response or resistance to lamivudine therapy among patients with different HBV genotypes.
Methods: Of 67 Japanese and Chinese patients with chronic hepatitis B, 18 patients with HBV/Bj, 15 with HBV/Ba and 34 with HBV/C were selected for a case–control study matched according to gender and age. All the patients were treated with lamivudine for 2 years and evaluated the response or emergence of the YMDD mutation at year 2 during the treatment. HBV genotypes were detected by the restriction fragment length polymorphism. The YMDD mutation was detected by the direct sequencing after amplification by PCR.
Results: At year 2 during therapy, 44.8% of the patients showed normalization of ALT and undetectable HBV DNA (favorable response), 35.8% developed the YMDD mutation. There was no significant difference of response to the therapy among the three genotype groups. The emergence of the YMDD mutation was associated with HBV/C. By the multiple logistic regression analysis, however, the significant factor of a favorable response was a higher pretreatment ALT level and negative HBeAg status and the significant factor of the emergence of the YMDD mutation was HBV/C.
Conclusions: Higher pretreatment ALT level, HBeAg status or HBV genotype may affect the response or resistance to lamivudine therapy.

Keywords: HBV genotype Ba, Bj, C; Lamivudine; Response to therapy; Resistant mutation

1. Introduction

Hepatitis B virus (HBV) is one of the major causative agents of acute or chronic liver diseases in Asian countries [1]. All strains of HBV isolated from various countries can be classified into eight HBV genotypes, HBV genotype A

Abbreviations: HBV/B, hepatitis B virus genotype B; HBV/Ba, hepatitis B virus subtype Ba; HBV/Bj, hepatitis B virus subtype Bj; HBV/C, hepatitis B virus genotype C

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(HBV/A) to HBV/H, according to their phylogenetic relationship [2–4]. As reported previously, patients with different HBV genotypes show genotype-specific manifestations in their clinical and virologic features [5–8]. In addition, geography-specific distributions of HBV genotypes have been demonstrated among areas and countries in world wide [5,7,9]. In south-east Asian countries, such as Japan, Taiwan or China, HBV/B and HBV/C are most prevalent [6,7,9]. Patients with HBV/B tend to have anti-HBe at a younger age and less chance to develop hepatocellular carcinoma compared to those with HBV/C [6–8,10].

Recently, we have demonstrated that HBV/B strains should be divided into two subtypes, HBV/Ba and HBV/Bj, according to their genetic relationship, and that HBV/Ba is found ubiquitously in Asian countries, while HBV/Bj is found only in Japan [11,12]. We also demonstrated that a higher proportion of patients with HBV/Ba have a HBeAg-positive status and hepatocellular carcinoma at a younger age than those with HBV/Bj [12,13].

Lamivudine is one of the widely available anti-virus drugs for patients with chronic HBV infection, which strongly suppress the reverse transcriptase of HBV. However, some patients develop unsatisfactory anti-viral effects during therapy because of the emergence of lamivudine-resistant strains during therapy [14,15]. It is still controversial whether the efficacy of lamivudine therapy may be associated with the difference of HBV genotypes. Therefore, the aim of this study was to investigate the response to lamivudine therapy for 2 years among patients with different genotypes of HBV/Ba, HBV/Bj or HBV/C.

2. Patients and methods

2.1. Patient selection

One hundred and twenty-two patients with chronic HBV infection were treated with lamivudine, 100 mg daily, for 2 years in various hospitals in Japan and Hong Kong. All patients were consecutively enrolled in this study, and informed consent was obtained. The patients with normal ALT levels (40 IU/L or less) before commencement of the treatment were excluded. All patients were positive for HBsAg for more than 6 months and negative for both anti-HCV and anti-HIV. All patients were tested by ultrasonography or CT scan to rule out decompensated cirrhosis or hepatocellular carcinoma. For the comparison of the response to lamivudine therapy among different genotypes, 18 patients with HBV/Bj, 15 patients with HBV/Ba and 34 patients with HBV/C were selected and matched according to gender and age for a case–control study. Liver biopsy was performed in the 45 patients who agreed.

For the HBeAg-positive patients, a complete response to lamivudine therapy was defined as the normalization of ALT, undetectable serum HBV DNA by the real-time PCR method, and seroconversion from HBeAg to anti-HBe at year 2 during therapy. For all the patients, including both the HBeAg-positive and -negative groups, a favorable response to lamivudine therapy was defined as the normalization of ALT and undetectable serum HBV DNA at year 2 during therapy.

Lamivudine resistance was defined as the emergence of the YMDD motif mutation (YIDD and/or YVDD) at year 2. Breakthrough hepatitis was designated when emergence of the YMDD mutation and re-elevation of ALT level and HBV DNA level were found.

2.2. Virologic assays

HBsAg, HBeAg and anti-HBe were tested by chemiluminescence enzyme immunoassay (CLEIA). The serum HBV DNA level in all the samples was quantified by the real-time PCR method (HBV RTD-Direct test, SRL Inc., Tokyo) in Nagoya City University. The detection limit of this assay was 100 copies/mL. The HBV genotype was determined by restriction fragment length polymorphism as described previously [16]. When the test results were inconclusive, the sequences of the S region were determined directly, then the genotype was decided by phylogenetic analysis [16,17]. The subtypes of HBV/Ba and HBV/Bj were also determined by restriction fragment length polymorphism [11]. The YMDD motif mutations (M204I/V) during lamivudine therapy were detected by the direct sequencing method after amplification by PCR. The Pre-C mutation at nucleotide position 1896 and the basal core promoter double mutations at nucleotide positions 1762 and 1764 were detected using the direct sequencing method [18].

2.3. Statistical analysis

The data were statistically analyzed by Student's t-test, the non-parametric Mann–Whitney test and the Chi-square test where appropriate. The multivariate analysis was accomplished by multiple logistic regression analysis. A p-value less than 0.05 was regarded as statistically significant.

3. Results

3.1. Baseline differences among the three HBV genotype groups

In the various baseline clinical and virologic factors, there were significant differences among the HBV/Bj, HBV/Ba and HBV/C groups, while age and gender were matched (Table 1). In the HBV/C group, a higher proportion of the patients at the F3 or F4 stage were found compared to the HBV/Bj or HBV/Ba group. The mean ALT level before treatment in the HBV/Ba group was significantly lower than the HBV/Bj or HBV/C group. Only 3% of the HBV/Bj group showed positive HBeAg, compared to 80.0% of the HBV/Ba group and 79.4% of the HBV/C group. The mean serum HBV

Table 1
Baseline clinical and virologic characteristics of the patients studied

Characteristics	HBV genotype			p-Value
	HBV/Bj ($n=18$)	HBV/Ba ($n=15$)	HBV/C ($n=34$)	
Gender (M:F)	18:0	15:0	34:0	Matched
Age (year, mean ± S.D.)	43.4 ± 11.4	40.5 ± 15.2	43.2 ± 8.9	Matched
Ethnic (Japanese:Chinese)	18:0	5:10	24:10	<0.01
Stage (F1:F2:F3:F4:ND)	10:0:2:0:6	4:1:1:0:9	6:6:7:8:7	<0.01
ALT (IU/L, mean ± S.D.)	293.3 ± 353.0	152.5 ± 130.8	309.9 ± 288.3	<0.05
Positive for HBeAg	3 (16.7%)	12 (80.0%)	27 (79.4%)	<0.01
HBV DNA level (log copies/mL, mean ± S.D.)	5.96 ± 1.69	8.17 ± 0.81	7.40 ± 1.09	<0.01
Pre-C (nt. 1896) mutation (wild:mutant)	4:14	10:5	26:8	<0.01
Core promoter (nt. 1762/1764) mutations (wild:mutant)	16:2	13:2	5:27 (ND:2)	<0.01

DNA level of the HBV/Bj group was significantly lower than the HBV/Ba or HBV/C group. A higher proportion of the HBV/Bj group had pre-C mutation compared to the HBV/Ba or HBV/C group. In contrast, a higher proportion of the HBV/C group had the core promoter mutations compared to the HBV/Bj or HBV/Ba group.

3.2. Response to lamivudine therapy

Of all the 67 patients, 44.8% showed a favorable response to lamivudine therapy (Fig. 1a). 61.1% of the HBV/Bj group, 40.0% of the HBV/Ba group and 38.2% of the HBV/C group showed a favorable response to the therapy, respectively. However, there was no significant difference in the favorable response rate among the different HBV genotype groups.

When the patients were stratified into the HBeAg-positive or -negative group, 33.3% showed complete response in the patients with positive HBeAg, compared to 60.0% in the negative HBeAg group ($p<0.05$; Fig. 1b). There was no significant difference in the favorable response rate among the three HBV genotype groups, even if the

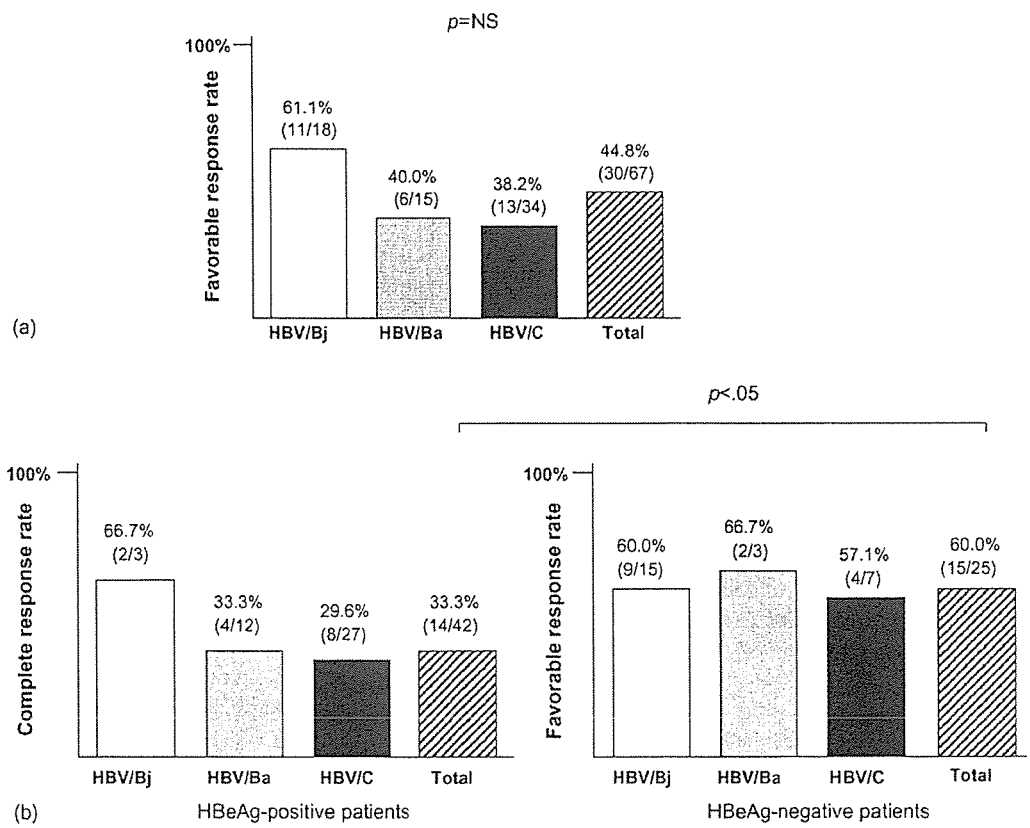

Fig. 1. The rate of favorable responses to lamivudine therapy in both HBeAg-positive and -negative patients among three different genotype groups (a) and the rate of complete response in the HBeAg-positive patients and the rate of favorable response in the HBeAg-negative patients (b).

Table 2
Complete response rate in HBeAg-positive patients with HBV/Ba and HBV/C

Characteristics	HBV genotype		p-Value
	HBV/Ba ($n=12$)	HBV/C ($n=27$)	
Age (years)			
<40	4/8 (50.0)	2/13 (15.4)	0.22
≥40	0/4 (0)	6/14 (42.9)	0.31
ALT (IU/L)			
<200	1/9 (11.1)*	1/9 (11.1)	0.45
≥200	3/3 (100)*	7/18 (38.9)	0.18
HBV DNA level (log copies/mL)			
<7.5	1/1 (100)	2/10 (20.0)	0.59
≥7.5	3/11 (27.3)	6/17 (35.3)	0.97
Pre-C (nt. 1896) mutation			
Wild	2/9 (22.2)	6/25 (24.9)*	0.72
Mutant	2/3 (66.7)	2/2 (100)*	0.81
Core promoter (nt. 1762/1764) mutations			
Wild	4/12 (33.3)	1/2 (50.0)	0.73
Mutant	0/0 (0)	7/23 (30.4)	–

Values in parenthesis are given in percentage.
* $p<0.05$.

patients were stratified into HBeAg-positive or -negative groups.

In the patients with positive HBeAg, pretreatment factors associated with complete response were analyzed in 12 patients in the HBV/Ba group and 27 in the HBV/C group (Table 2). Between the two genotype groups, there were no significant differences of the factors predicting complete response to the therapy in age, ALT level, HBV DNA level, pre-C mutation and core promoter mutations. However, in the HBV/Ba group, the patients who had 200 IU/L or higher ALT levels showed complete response more frequently than those with less than 200 IU/L. In the HBV/C group, a higher proportion of the patients with pre-C mutation tended to have a complete response compared to those with wild pre-C.

Of the patients with negative HBeAg, 15 in the HBV/Bj group and 7 in the HBV/C group, there were no significant differences in the predicting factors for a favorable response to the therapy between the HBV/Bj and HBV/C groups (Table 3).

To investigate the significance of predicting factors associated with complete response to lamivudine therapy in HBeAg-positive patients, stepwise logistic regression analysis was performed (Table 4). A factor of the pretreatment ALT level of 200 IU/L or higher and pre-C mutation were significant factors (odds ratio: 12.056, $p=0.03$ and 25.553, $p=0.03$, respectively).

In all the patients with both positive and negative HBeAg, the significant factors predicting a favorable response to the therapy were a pretreatment ALT level of 200 IU/L or higher (odds ratio: 3.715, $p=0.02$) and a negative HBeAg status (odds ratio: 3.472, $p=0.03$; Table 5). The other factors were not significant.

Table 3
Favorable response rate in HBeAg-negative patients with HBV/Bj and HBV/C

Characteristics	HBV genotype		p-Value
	HBV/Bj ($n=15$)	HBV/C ($n=7$)	
Age (years)			
<40	1/2 (50.0)	1/1 (100)	0.67
≥40	8/13 (61.5)	3/6 (50.0)	0.98
ALT (IU/L)			
<200	4/8 (50.0)	1/3 (33.3)	0.85
≥200	5/7 (71.4)	3/4 (75.0)	0.56
HBV DNA level (log copies/mL)			
<7.5	7/13 (53.8)	1/4 (25.0)	0.66
≥7.5	2/2 (100)	3/3 (100)	–
Pre-C (nt. 1896) mutation			
Wild	2/9 (22.2)	1/1 (100)	0.65
Mutant	7/13 (53.8)	3/6 (50.0)	0.74
Core promoter (nt. 1762/1764) mutations			
Wild	7/13 (53.8)	2/3 (66.7)	0.81
Mutant	2/2 (100)	2/4 (50.0)	0.76

Values in parenthesis are given in percentage.

Table 4
Significant factors associated with complete response to lamivudine therapy in HBeAg-positive patients by the stepwise logistic regression analysis

Factors	Odds ratio	95% CI	p-Value
ALT level (IU/L)			
<200	1		
≥200	12.056	1.322–109.907	0.03
Pre-C (nt. 1896) mutation			
Wild	1		
Mutant	25.553	1.391–469.526	0.03

3.3. Emergence of YMDD mutation

Of all the patients, 35.8% emerged the YMDD mutation at year 2 during therapy (Fig. 2a). In the HBV/C group, 50.0% had the YMDD mutation compared to 27.8% in the HBV/Bj and 13.3% in the HBV/Ba group ($p<0.05$, respectively). When the patients were stratified into positive or negative HBeAg status, 51.9% of the HBV/C group with positive HBeAg emerged the YMDD mutation compared to 8.3% of the HBV/Ba group with positive HBeAg, even though HBeAg positivity and HBV DNA levels at baseline

Table 5
Significant factors associated with favorable response to lamivudine therapy in both HBeAg-positive and -negative patients by the logistic regression analysis

Factors	Odds ratio	95% CI	p-Value
ALT level (IU/L)			
<200	1		
≥200	3.715	1.269–10.875	0.02
HBeAg status			
Positive	1		
Negative	3.472	1.156–10.417	0.03

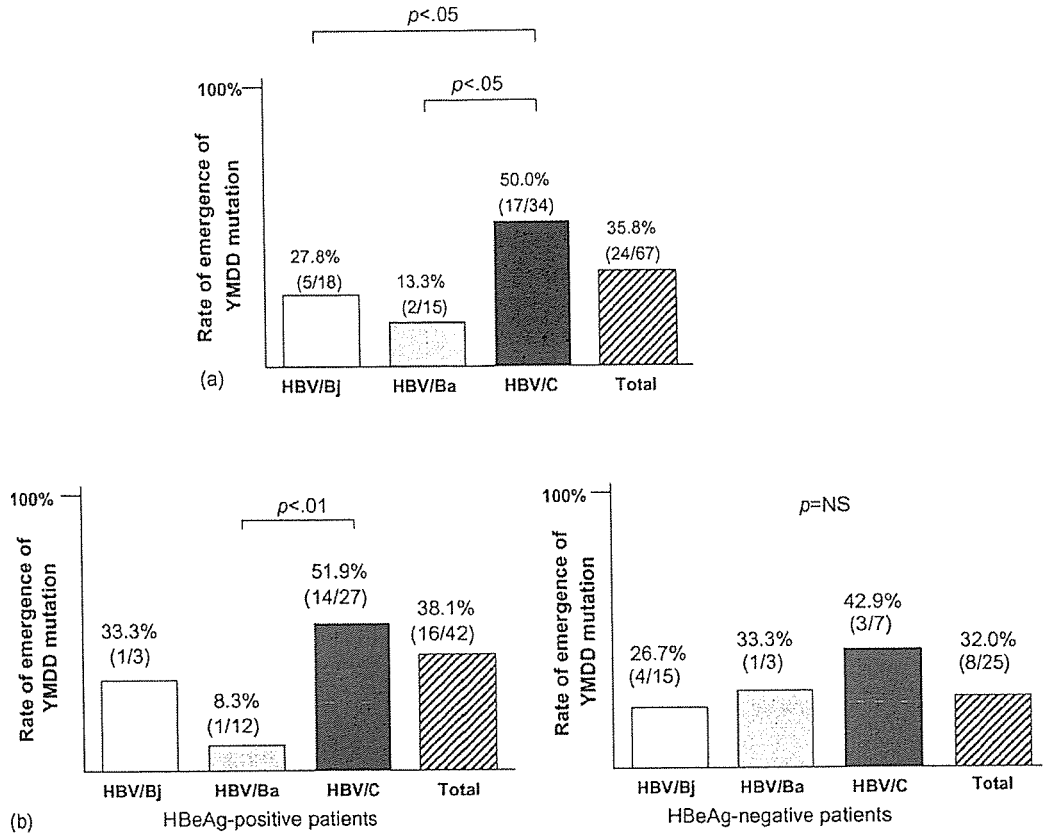

Fig. 2. The emergence rate of the YMDD mutation among three different genotype groups (a) and the emergence rate of the YMDD mutation stratified into HBeAg-positive and -negative patients (b).

were almost same between the HBV/Ba and HBV/C groups ($p < 0.01$; Fig. 2b). There was a trend for a higher proportion of the HBV/C group to have the YMDD mutation in the patients with negative HBeAg. However, the statistical differences were obscure because the numbers of the patients were small when they were stratified into HBeAg-positive or -negative groups.

The pretreatment factors predicting the emergence of the YMDD mutation were analyzed among three HBV genotype groups (Table 6). In the patients aged less than 40 years, 57.1% in the HBV/C group emerged the YMDD mutation compared to 12.5% in the HBV/Ba group ($p < 0.05$). In the patients with wild pre-C, the YMDD mutation emerged more frequently in the HBV/C group than in the HBV/Ba group ($p < 0.05$). In the patients with 7.5 or more log copies/mL of the HBV DNA level, the YMDD mutation emerged more frequently in the HBV/C group than in the HBV/Ba group ($p < 0.05$). Stratified in the positive HBeAg group, 58.9% in the HBV/C group with 7.5 or more log copies/mL of the HBV DNA level emerged the YMDD mutation compared to 40.0% in the HBV/C group with less than 7.5 log copies/mL of the HBV DNA level. However, in the HBV/Ba group with positive HBeAg, there was no difference of the frequency of the YMDD mutation between the higher or the lower HBV DNA groups.

The significant factor predicting emergence of the YMDD mutation by the stepwise logistic regression analysis was HBV/C, compared to HBV/Bj or HBV/Ba (odds ratio: 3.714, $p = 0.02$; Table 7). The other factors such as HBeAg status, HBV DNA levels and ALT levels at baseline were not significant.

3.4. Breakthrough hepatitis

Of all the patients, 11.9% had breakthrough hepatitis at year 2 during therapy (Fig. 3). There was no significant difference in the frequency of breakthrough hepatitis among the three HBV genotype groups.

4. Discussion

It is well-known that the clinical and virologic manifestations of patients with chronic HBV infection depend upon their HBV genotypes [5–8]. However, it is still controversial whether the response or resistance to lamivudine therapy is associated with HBV genotypes. To analyze the relationship between the response or resistance to lamivudine therapy and pretreatment factors, for the first time we conducted an age and gender matched case–control study among Japanese and

Table 6
Emergence of the YMDD mutation in patients among three HBV genotype groups

Characteristics	HBV genotype			p
	HBV/Bj (n=18)	HBV/Ba (n=15)	HBV/C (n=34)	
Age (year)				
<40	2/5 (40.0%)	1/8 (12.5%) *	8/14 (57.1%)	<.05
≥40	3/13 (23.1%)	1/7 (14.3%)	9/20 (45.0%)	.22
ALT				
<200	3/11 (27.3%)	2/11 (18.2%) *	8/12 (66.7%)	<.05
≥200	2/7 (28.6%)	0/4 (0%)	9/22 (40.9%)	.26
HBV DNA level (log copies/mL)				
<7.5	4/15 (26.7%)	1/2 (50.0%)	7/14 (50.0%)	.41
≥7.5	1/3 (33.3%)	1/13 (7.7%) *	10/20 (50.0%)	<.01
Pre-C (nt.1896) mutation				
Wild	0/4 (0%)	1/10 (10.0%) *	14/26 (53.8%)	<.05
Mutant	5/14 (35.7%)	1/5 (20.0%)	3/8 (37.5%)	.77
Core promoter (nt.1762/1764) mutations				
Wild	5/16 (31.3%)	1/13 (7.7%)	1/5 (20.0%)	.30
Mutant	0/2 (0%)	1/2 (50.0%)	15/27 (55.6%)	.31

*$p<0.05$

Table 7
Significant factors associated with emergence of the YMDD mutation by the stepwise logistic regression analysis

Factors	Odds ratio	95% CI	p-Value
HBV genotype			
Bj or Ba	1		
C	3.714	1.272–10.847	0.02

Chinese patients with HBV genotypes Bj, Ba and C. In this study, it was indicated that the significant factors predicting a favorable response to lamivudine therapy do not include the HBV genotype, but a higher pretreatment ALT level or negative HBeAg status and that the significant factor predicting emergence of the YMDD mutation is the HBV genotype (HBV/C).

The natural course of chronic HBV infection is usually affected by age and gender [19]. The natural seroconversion from positive HBeAg to anti-HBe is sometimes observed with the normalization of ALT in younger adults [8,10]. In addition, females often experience seroconversion after delivery [20]. So, age and sex are major factors influencing the natural course of chronic HBV infection. Thus, this report is a first case–control study to investigate the relationship between HBV genotype and response to lamivudine therapy.

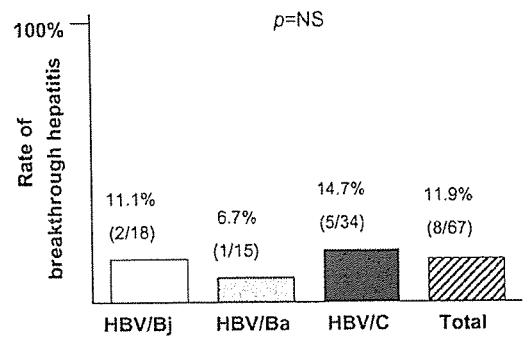

Fig. 3. The rate of breakthrough hepatitis among the three different genotype groups.

In all the patients, there was no significant difference of the rate of favorable response to the therapy among the three HBV genotype groups, even if the patients were stratified into an HBeAg-positive or -negative status. Although this study was an age and gender matched case–control study, the frequency of positive HBeAg was very low in the HBV/Bj group compared with the HBV/Ba or HBV/C groups because a higher proportion of patients with HBV/Bj have negative HBeAg status compared to those with HBV/Ba or HBV/C, as previously reported [12]. So, to investigate the relationship of the HBV genotype with the response to the therapy, we also compared the positive HBeAg patients between the HBV/Ba and HBV/C group. There was no significant difference in the response to the therapy between two groups. In addition, in the patients with negative HBeAg, there was also no significant difference of response to the therapy between the HBV/Bj and HBV/C group. The multiple logistic regression analysis revealed that a higher pretreatment ALT level and pre-C mutation were significant factors predicting complete response in the patients with positive HBeAg, and that higher ALT level and negative HBeAg status were significant factors predicting favorable response in all the patients.

Recently, some papers have reported the relationship between the HBV genotype and the response to lamivudine therapy, while the other paper denied this relationship [21–27]. Therefore, it is still controversial whether the

HBV genotype is associated with the response to lamivudine therapy for more than 1 year. Thus, we conducted a case–control study which revealed no association of HBV genotype with response to therapy. However, there remained to be a significant bias in the baseline features, such as a positive rate of HBeAg. It is ideal that age, gender and HBeAg status are matched. However, there are very few adult patients with HBV/Bj who are positive HBeAg and still have indication for lamivudine therapy, since those patients with HBV/Bj tend to experience seroconversion at a younger age. So, each paper might conduct various results in different backgrounds.

A higher pretreatment ALT level was one of the important predictors for a complete response to lamivudine therapy [28,29]. In this study, an ALT level greater than 200 IU/L and the pre-C mutation were significant factors in the patients with positive HBeAg. It was considered that the pre-C mutation is associated with the induction of a negative HBeAg status [30]. In all the patients, a higher ALT level and a negative HBeAg status were predicting factors for a favorable response, as previously reported [31,32].

It was reported previously that higher HBV DNA level or positive HBeAg status were significant predicting factors for the emergence of the YMDD mutation among the consecutive patients with HBV genotypes A–C in the cross-section studies [22,23]. In general, a higher proportion of Japanese patients with HBV/C tend to have a higher HBV DNA level and a positive HBeAg status which may lead a trend of emergence of the YMDD mutation [22,29,32]. In this study, there was also a trend of higher frequency of emergence of the YMDD mutation in the HBV/C group with positive HBeAg and a higher HBV DNA level. However, the significant predicting factor of emergence of the YMDD mutation was HBV genotype (HBV/C) by the multivariate analysis in the present study. It is still unclear why the viral factors, such as genotype, HBV DNA level or HBeAg status, may affect the chance of the emergence of the YMDD mutation. Further in vitro or vivo studies are warranted.

In this study, the number of patients (only eight) who developed breakthrough hepatitis was too small to investigate the factors affecting breakthrough hepatitis. If we were to observe this cohort of patients for a longer term, we may investigate more information, such as the L180M mutation or other mutations of the HBV genome [33].

The factors associated with a favorable response to lamivudine therapy or the emergence of the YMDD mutation may vary according to the baseline features of the patients studied. In this case–control study, a higher ALT level and negative HBeAg status were significant factors predicting a favorable response to lamivudine therapy, and HBV genotype C was a significant factor predicting the emergence of the YMDD mutation.

However, the patient numbers of this case–control study were not enough to confirm these results. Thus, further studies are warranted with a large number of the patients in each HBV genotype group.

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References

[1] Lee WM. Hepatitis B virus infection. N Engl J Med 1997; 337:1733–45.
[2] Okamoto H, Tsuda F, Sakugawa H, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. J Gen Virol 1988;69:2575–83.
[3] Stuyver L, Gendt S, Van Geyt C, et al. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67–74.
[4] Arauz-Ruiz P, Norder H, Robertson BH, et al. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. J Gen Virol 2002;83:2059–73.
[5] Lindh M, Andersson AS, Gusdal A. Genotypes, nt 1858 variants, and geographic origin of hepatitis B virus large-scale analysis using a new genotyping method. J Infect Dis 1997;175:1285–93.
[6] Kao JH, Chen PJ, Lai MY, et al. Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. Gastroenterology 2000;118:554–9.
[7] Orito E, Ichida T, Sakugawa H, et al. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. Hepatology 2001;34:590–4.
[8] Orito E, Mizokami M, Sakugawa H, et al. A case–control study for clinical and molecular biological differences between hepatitis B virus of genotypes B and C. Hepatology 2001;33:218–23.
[9] Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. Intervirology 2003;46:329–38.
[10] Yuen MF, Sablon E, Tanaka Y, et al. Epidemiological study of hepatitis B virus genotypes, core promoter and precore mutations of chronic hepatitis B infection in Hong Kong. J Hepatol 2004;41:119–25.
[11] Sugauchi F, Orito E, Ichida T, et al. Hepatitis B virus genotype B with or without recombination with genotype C over the precore region plus the core gene. J Virol 2002;76:5985–92.
[12] 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;124:925–32.
[13] Orito E, Sugauchi F, Tanaka Y, et al. Differences of hepatocellular carcinoma patients with hepatitis B virus genotypes of Ba, Bj or C in Japan. Intervirology 2005;48:239–45.

[14] Lai CL, Chien RN, Leung NWY, et al. A one-year trial of lamivudine for chronic hepatitis B. N Engl J Med 1998;339:61–8.
[15] Chayama K, Suzuki Y, Kobayashi M, et al. Emergence and takeover of YMDD motif mutant hepatitis B virus during long-term lamivudine therapy and re-takeover by wild type after cessation of therapy. Hepatology 1998;27:1711–6.
[16] Mizokami M, Nakano T, Orito E, et al. Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. FEBS Lett 1999;450:66–71.
[17] Ohba K, Mizokami M, Ohno T, et al. Relationships between serotypes and genotypes of Hepatitis B virus genetic classification. Virus Res 1995;39:25–34.
[18] Chan HI, Hussain M, Lok AS. Different hepatitis B virus genotypes are associated with different mutations in the core promoter and precore regions during hepatitis B e antigen seroconversion. Hepatology 1999;29:976–84.
[19] Lok AS. Natural history and control of perinatally acquired hepatitis B virus infection. Dig Dis 1992;10:46–52.
[20] Lin HH, Chen PJ, Chen DS, et al. Postpartum subsidence of hepatitis B viral replication in HBeAg-positive carrier mothers. J Med Virol 1989;29:1–6.
[21] Chien RN, Yeh CT, Tsai SL, et al. Determinants for sustained HBeAg response to lamivudine therapy. Hepatology 2003; 38:1267–73.
[22] Yuen MF, Wong DK, Sablon E, et al. Hepatitis B virus genotypes B and C do not affect the antiviral response to lamivudine. Antivir Ther 2003;8:531–4.
[23] Suzuki F, Tsubota A, Arase Y, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. Intervirology 2003;46: 182–9.
[24] Akuta N, Suzuki F, Kobayashi M, et al. The influence of hepatitis B virus genotype on the development of lamivudine resistance during long-term treatment. J Hepatol 2003;38:315–21.
[25] Zollner B, Petersen J, Puchhammer-Stockl E, et al. Viral features of lamivudine resistant hepatitis B genotypes A and D. Hepatology 2004;39:42–50.
[26] Moskovitz DN, Osiowy C, Giles E, et al. Response to long-term lamivudine treatment (up to 5 years) in patients with severe chronic hepatitis B, role of genotype and drug resistance. J Viral Hepat 2005;12:398–404.
[27] Lo CM, Cheung CK, Lau GK, et al. Significance of hepatitis B virus genotype in liver transplantation for chronic hepatitis B. Am J Transplant 2005;5:1893–900.
[28] Leung NWY, Lai CL, Chang TT, et al. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology 2001;33:1527–32.
[29] Perrillo RP, Lai CL, Liaw YF, et al. Predictors of HBeAg loss after lamivudine treatment for chronic hepatitis B. Hepatology 2002;36:186–94.
[30] Laras A, Koskinas J, Avgidis K, et al. Incidence and clinical significance of hepatitis B virus precore gene translation initiation mutations in e antigen-negative patients. J Viral Hepat 1998;5:241–8.
[31] Lau DTY, Khokhar MF, Doo E, et al. Long-term therapy of chronic hepatitis B with lamivudine. Hepatology 2000;32:828–34.
[32] Chang ML, Chien RN, Yeh CT, et al. Virus and transaminase levels determine the emergence of drug resistance during long-term lamivudine therapy in chronic hepatitis B. J Hepatol 2005;43:72–7.
[33] Tanaka Y, Yeo AET, Orito E, et al. Prognostic indicators of breakthrough hepatitis during lamivudine monotherapy for chronic hepatitis B virus infection. J Gastroenterol 2004;39:769–75.

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Spatial and chronological differences in hepatitis B virus genotypes from patients with acute hepatitis B in Japan

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Abstract

Genotypes of hepatitis B virus (HBV) were determined in 485 patients with acute hepatitis B from all over Japan. They were A in 92 (19%), Ba in 26 (5%), Bj in 32 (7%), C in 330 (68%) and D in 5 (1%). Sexual contacts were the main route of transmission in them. Overall,

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HBV persisted in only 5 of the 464 (1%) followed patients. Genotypes C accounted for more than 68% in northern as well as southern areas, contrasting with genotype A accounting for 34% in and around the Metropolitan areas. During 24 years from 1982 to 2005, genotype A increased from 5% to 33%, while genotype B gradually decreased from 26% to 8%. Fulminant hepatitis was significantly more frequent in infection with genotype Bj (41%) than those with the other genotypes ($p < 0.01$). The core-promoter double mutation (T1762/A1764) and precore stop-codon mutation (A1896) were more frequent in patients with fulminant than acute self-limited hepatitis (57% versus 15% and 58% versus 10%, respectively, $p < 0.01$ for both). In conclusion, genotype A distributes unevenly over Japan, prevails in younger patients through sexual transmission and has increased with years. Furthermore, fulminant outcome was more frequent in patients with genotype Bj than those with the other genotypes.

Keywords: Chronic hepatitis; Fulminant hepatitis; Hepatitis B e antigen; Hepatitis B surface antigen; Sexual transmission

1. Introduction

Mass vaccination with plasma-derived or recombinant vaccines has been effective in Asian countries hyperendemic with hepatitis B virus (HBV), as well as in the United States and France. In Japan, perinatal transmission of HBV from mothers with hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg) in serum used to be the principal route to establish the persistent carrier state [1]. Hence, passive and active immunoprophylaxis of babies born to carrier mothers with HBeAg by combined hepatitis B immunoglobulin and vaccine was mandated since 1986, and extended to carrier mothers without HBeAg in 1995. As the results, HBsAg has become rare in the Japanese born after 1986, and is detected in merely 0.3% of the first-time blood donors younger than 19 years at present [2].

There is an increasing trend, however, for acute HBV infection to occur preferentially in young men through promiscuous sexual contacts [3–7]. Foreign sexual workers from neighboring Asian countries are suspected as significant sources of de novo HBV infection in them [4,8]. Thus, patients with acute hepatitis visiting hospitals are increasing all over Japan. Since the majority of acute HBV infections ran subclinical courses, hospitalized cases of acute hepatitis B would represent the tip of an iceberg.

Eight genotypes have been detected by the sequence divergence >8% in the entire HBV genome composed of approximately 3200 nucleotides (nt), and designated by capital alphabet letters from A to H in the order of documentation [9–12]. They have distinct geographical distribution and are associated with severity of liver disease as well as response to antiviral therapies [13–15]. Furthermore, subgenotypes have been reported for HBV/A, B and C, and named Aa (Asian/African type) and Ae (European type) [16], Bj (Japanese type) and Ba (Asian type) [17], as well as Ce (east Asian type) and Cs (southeast Asian type) [18]. There have been increasing lines of evidence that Aa and Ae, as well as Ba and Bj, influence the replication of HBV and bear clinical relevance [19–21].

Over 24 years from 1982 to 2005, a multicenter study was conducted throughout Japan on 547 patients with acute hepatitis B. Genotypes were determined on viral isolates recovered from them, and analyzed for distributions changing with time and in place. The results highlighted foreign HBV genotypes represented by HBV/A which have increased through sexual contacts, and HBV/Bj prevalent in patients with fulminant hepatitis.

2. Patients and methods

2.1. Patients with acute hepatitis B

During 1982 through 2005, 547 patients with acute hepatitis B were registered in 25 hospitals throughout Japan, of them, 147 and 336 cases are overlapping with previous report from Yotsuyanagi et al. [7] and Ozasa et al. [22], respectively, and 64 cases were newly registered in this study. These hospitals were grouped into the following eight areas: Hokkaido, Tohoku, Kanto, Koshin, Tokai, Kinki, Chugoku/Shikoku and Kyushu/Okinawa. The diagnosis of acute hepatitis B was contingent on a sudden onset of clinical symptoms of hepatitis and detection of high-titered antibody to hepatitis B core antigen (anti-HBc) of IgM class in serum. The great majority of them were followed for clinical outcomes until the disappearance of HBsAg through 24 weeks or longer after the presentation. HBV genotypes were determined in sera stored at −40 °C, and their geographical distributions and chronological changes were analyzed. Further, they were correlated with sources of infection and clinical outcomes of acute hepatitis. The study protocol conformed to the 1975 declaration of Helsinki, and was approved by Ethics Committees of institutions. Every patient or his/her next of kin gave an informed consent on the purpose of this study.

2.2. Serological markers of HBV infection

HBsAg was determined by hemagglutination (MyCell, Institute of Immunology Co. Ltd., Tokyo, Japan) or enzyme-linked immunosorbent assay (ELISA) (AxSYM, Abbott Japan, Tokyo, Japan), and HBeAg by ELISA (ELISA, F-HBe, Kokusai Diagnostic, Kobe, Japan). Anti-HBc of IgM class was determined by ELISA (HBc-antiM RIA, Dainabot, Tokyo, Japan).

2.3. Genotypes and subgenotypes of HBV

The six major HBV genotypes (A–F) were determined serologically by ELISA using commercial kits (HBV GENO-

TYPE EIA, Institute of Immunology). The method depends on the combination of epitopes on preS2-region products detected by monoclonal antibodies that is specific for each of them [23,24]. Genotypes were confirmed by restriction fragment length polymporphisim (RFLP) when required [25].

HBV/Bj (Japanese type) without the recombination with genotype C over the precore region and the core gene and Ba (Asian type) with the recombination were determined by its absence or presence on HBV DNA sequences, as well as RFLP involving on specific nucleotide substitutions, by the method described previously [26].

2.4. Point mutations in the precore resion and basic core-promoter (BCP)

Mutations in the precore region for A1896 and BCP for T1762/A1764 were detected by enzyme-linked minisequence assay (Smitest HBV Pre-C ELMA, Roche Diagnostic, Tokyo, Japan) according to the manufacturer's instructions, or by sequencing the precore region and BCP using the method described previously [27]. The results were recorded as "the wild-type" or "the mutant-type" expressed dominantly by HBV isolates.

2.5. Statistical analysis

Categorial variables were compared between groups by the χ^2-test or Fisher's exact test, and non-categorial variables by the Mann–Whitney's U-test. A p-value less than 0.05 was considered significant.

3. Results

3.1. Clinical profiles of patients with acute hepatitis B

During 1982 through 2005, 547 patients with acute hepatitis B were registered in 25 hospitals from all over Japan. Genotypes of HBV were unclassifiable in 40 (7%) and sufficient clinical data not available in 22 (4%) of them. Exclusive of these 62 patients, 485 (89%) were left for the evaluation of geographic distribution of HBV genotypes, as well as their changes with time, transmission routes and relevance with clinical outcomes.

The 485 patients with acute hepatitis B had the mean ± S.D. age of 35.7 ± 13.7 years, and included 338 (70%) men. Their peak alanine aminotransferase (ALT) averaged 2576 ± 1673 IU/L and peak total bilirubin 9.5 ± 9.5 mg/dL. They all possessed anti-HBc of IgM class in high titers, and HBeAg was detected in sera from 338 (70%) of them at the presentation. Fulminant hepatitis with coma of grade >II and prothrombin time <40% developed within 8 weeks after the onset in 45 (9%) of them.

Fulminant hepatitis led to death in 18 (5%) patients, and 3 (1%) received liver transplantation. Exclusive of these 21 patients, HBV persisted in only 5 of the 464 (1%) studied patients with acute hepatitis. They represented 3 of the 92 (3%) infected with HBV/A, 1 of the 58 (2%) with B and 1 of the 330 (0.3%) with C. None of the five patients with chronic outcome had received antiviral or steroid treatment during their acute phase of illness.

3.2. Geographic distribution of HBV genotypes in patients with acute hepatitis B

Overall, HBV/A was detected in 92 (19%), Ba in 26 (5%), Bj in 32 (7%), C in 330 (68%) and D in 5 (1%). Distribution of HBV genotypes over Japan is illustrated in Fig. 1. They dispersed unevenly in place. HBV/C accounted for more than 68% in both northern and southern areas, contrasting with HBV/A accounting for 34% in and around the Metropolitan area. Among HBV/B infections, the proportion of HBV/Ba was higher in Koshin, Kinki and Kyushu/Okinawa (14/17, 1/1 and 2/3), while that of HBV/Bj was higher in Hokkaido, Tohoku, Kanto and Tokai (4/4, 6/7, 13/21 and 5/5).

3.3. Demographic and clinical differences of patients infected with various genotypes

Table 1 compares demographic and clinical characteristics of patients with different HBV genotypes. Patients with HBV/D were excluded from the analysis due to their small numbers. The mean age was lower in patients with HBV/A (31.8 ± 10.9 years) than HBV/Ba (40.7 ± 10.9, $p < 0.01$), HBV/Bj (41.2 ± 17.0, $p = 0.01$) and HBV/C (35.8 ± 13.9, $p < 0.03$); it was higher in patients with HBV/Ba than HBV/C (40.7 ± 10.9 versus 35.8 ± 13.9, $p = 0.02$). The proportion of patients aged <30 years was significantly greater in HBV/A (58%) than HBV/Ba (12%, $p < 0.01$), HBV/Bj (38%, $p < 0.04$) or HBV/C infection (42%, $p < 0.01$). Men predominated ($p < 0.01$) in infections with HBV/A and HBV/Ba (92% and 88%, respectively) than those with HBV/Bj and HBV/C (56% and 64%, respectively). The peak ALT level was higher in HBV/Bj (3371 ± 2342 IU/L) than HBV/A (2051 ± 1009, $p = 0.04$) or HBV/C (2650 ± 1747, $p < 0.03$) infection. HBeAg was detected in 84% of patients with HBV/A at the frequency much higher than that in those with HBV/Ba (54%, $p < 0.01$), HBV/Bj (59%, $p < 0.01$) or HBV/C (60%, $p < 0.01$).

The routes of transmission were sexual contacts in 216 (45%) patients, followed by medical accidents in 14 (8%), blood transfusion in 4 (1%) and drug in 1 (0.2%); transmission routes were not identified in the remaining 245 (51%) patients. Sexual transmission was the most frequent cause of infection in 57% of HBV/A, 73% of HBV/Ba, 34% of HBV/Bj and 40% of HBV/C infections.

Fulminant hepatitis was significantly more frequent in patients infected with HBV/Bj (41%) than the other genotypes ($p < 0.01$); it occurred in 2 of the 5 (40%) patients with HBV/D, also. In reflection of severe clinical courses, the peak ALT level tended to be high in patients with HBV/Bj.