Table 3. Factors associated with survival for liver-related death in patients infected with HCV, who had not received antiviral therapy, identified by multivariate analysis

[Factors]	[Category]	Hazard ratio (95% confidence interval)	P
Gender	1: female	1	
	2: male	1.91 (1.45-2.52)	<0.001
Age (years)	1:<60	1	
	2:≥60	1.61 (1.21-2.12)	0.001
Albumin (g/dl)	1:≥3,9	1	
	2:<3.9	2.49 (1.87-3.31)	<0.001
Platelet count ($\times 10^4/mm^3$)	1:≥15.0	1	
	2:<15.0	3.69 (2.65-5.13)	<0.001
Aspartate aminotransferase (IU/l)	1:<67	1	
	2:≥67	4.16 (2.43-7.11)	<0.001
HCV subgroup	1: HCV-2a/2b	1	
	2: HCV-1b with Arg70	1.83 (1.25-2.68)	0.002
	3: HCV-1b with Gln70(His70)	2.16 (1.48-3.16)	<0.001

Cox proportional hazard model

the end of 5 years; 9.1, 0% at the end of 10 years; 20.5, 0% at the end of 15 years; and 20.5, 0% at the end of 20 years, respectively. The cumulative change rates in TT genotype were not significantly higher than those in non-TT genotype ($P = 0.114$) (Fig. 3B).

Discussion

This is the first report to indicate that aa substitution in the core region might affect hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. The treatment-resistant mechanism and oncogenic potential of HCV core region are still unclear. Moriishi et al.[28,29] showed that a knockout of the PA28γ gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of HCV core gene transgenic mice and disrupts development of both hepatic steatosis and HCC. Hu et al.[13] indicated that the point-mutations of the core gene, including core aa 70 and aa 91, might change the secondary structure of not only RNA but also protein. As a result, the functions of both RNA and protein of the core region, such as an interaction with other DNA/RNA or proteins, might change and lead to hepatocarcinogenesis. Funaoka et al.[30] recently reported that treatment-resistant substitutions of core aa 70 and aa 91 (Gln70/His70 and Met91) were resistant to interferon *in vitro*, and the resistance might be induced by interleukin 6-induced upregulation of SOCS3. Further studies should be performed to investigate the treatment-resistant mechanism and oncogenic potential of aa substitution in the core region.

The association between HCV genotype and the risk of HCC is not clear. A previous report indicated that hepatocarcinogenesis rates in patients infected with HCV-1b were significantly higher than those in patients infected with HCV-2a/2c, based on an Italian cohort,[31] and this finding might be partly explained by distribution of HCV-1b of Arg70 or Gln70(His70). In fact, the hepatocarcinogenesis rates in HCV-1b of Gln70(His70) were significantly higher than those in HCV-1b of Arg70 and HCV-2a/2b in the present study based on a Japanese cohort. The present study is the first report to indicate that substitution of aa 70 in

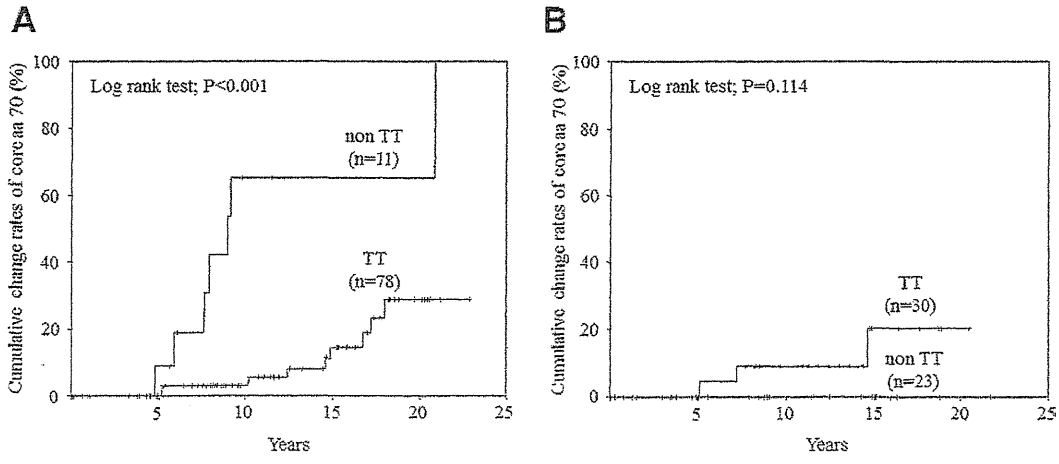

Fig. 3. Changes over time of dominant amino acid by direct sequencing in core aa 70 of HCV-1b, according to *IL28B* rs8099917 genotype. (A) In HCV-1b patients of Arg70 at the initial visit, cumulative change rates from Arg70 to Gln70(His70) during follow-up. The rates in non-TT genotype were significantly higher than those in TT genotype ($P < 0.001$; log-rank test). (B) In HCV-1b patients of Gln70(His70) at the initial visit, cumulative change rates from Gln70(His70) to Arg70 during follow-up. The rates in TT genotype were not significantly higher than those in non-TT genotype ($P = 0.114$; log-rank test).

the core region of HCV-1b is not only an important predictor of hepatocarcinogenesis, but also of survival for liver-related death in HCV patients who had not received antiviral therapy. The reason for the higher rates of liver-related death in HCV-1b of Gln70(His70) might be due to the higher rates of HCC. In conclusion, reducing the risk of hepatocarcinogenesis by HCV RNA eradication and/or ALT normalization by antiviral therapy should be recommended, especially in HCV-1b of Gln70(His70) as a high-risk group for hepatocarcinogenesis.[32]

The significant linkage between substitution of aa 70 and *IL28B* genotype had been shown,[21-23] but the mechanism of complex interaction between the virus and host is not clear. In the present study, the cumulative change rates from Arg70 to Gln70(His70) were significantly higher than those from Gln70(His70) to Arg70. Especially, the rates from Arg70 to Gln70(His70) in *IL28B* rs8099917 non-TT genotype were significantly higher than those in TT genotype. Although the molecular mechanisms of their relationship remain unknown, it could be speculated that *IL28B* genotype has an influence on the time-dependent changes of core aa 70, and refractory factors for treatment might accumulate in HCV-1b patients with non-TT. Hence, elucidating the relationship between substitution of aa 70 and *IL28B* genotype is an important step in understanding the mechanism of HCV treatment-resistance and disease progression.

The impact of *IL28B* genotype on hepatocarcinogenesis is controversial.[18-21] In this study, the effect of *IL28B* rs8099917 genotype on HCC was assessed in 515 of 2,799 consecutive HCV-infected patients who had not received antiviral therapy. Interestingly, the cumulative hepatocarcinogenesis rates in TT of the treatment-sensitive genotype was not significantly lower than those in non-TT of the treatment-resistant genotype ($P = 0.930$; log-rank test) in a preliminary study based on a small numbers of patients (Fig. 4). This result suggests that core aa 70 as a predictor of hepatocarcinogenesis might not only be influenced by *IL28B* genotype, but also by other factors strongly related to hepatocarcinogenesis independent of *IL28B* genotype. As a whole, it is regrettable that its impact on hepatocarcinogenesis in HCV patients who had not received antiviral therapy could not be investigated in this study. Further comprehensive studies should be performed to disclose the molecular mechanisms for the complicated relationships among core aa 70, *IL28B* genotype, and hepatocarcinogenesis.

The limitations of the present study are that patients who had received treatment besides IFN-related therapy (such as ursodeoxycholic acid, branched chain amino acid, and phlebotomy) could not be excluded. Furthermore, the clinical impact of metabolic factors (such as diabetes, insulin resistance, hepatocyte steatosis, and obesity) on hepatocarcinogenesis could also not be investigated. Further studies should be performed to investigate the clinical impact of treatment besides IFN-related therapy and metabolic factors on hepatocarcinogenesis.[33-37]

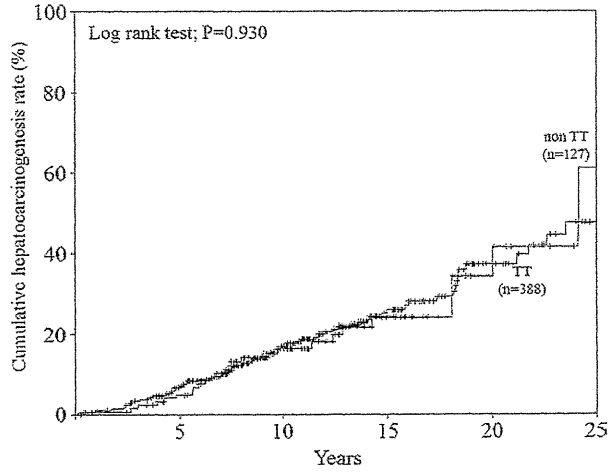

Fig. 4. Cumulative hepatocarcinogenesis rates according to *IL28B* rs8099917 genotype. The rates in TT genotype were not significantly lower than those in non-TT genotype ($P = 0.930$; log-rank test) in a preliminary study based on a small number of 515 patients.

In conclusion, substitution of aa 70 in the core region of HCV-1b is the important predictor of hepatocarcinogenesis and survival for liver-related death in HCV patients who had not received antiviral therapy. This study emphasizes the importance of antiviral therapy to reduce the risk of hepatocarcinogenesis, especially in HCV-1b of Gln70(His70) as a high-risk group for hepatocarcinogenesis. Furthermore, *IL28B* genotype might partly affect changes over time of dominant amino acid in core aa 70. This result should be interpreted with caution because races other than Japanese populations and patients infected with HCV-1a were not included. Any generalization of the results should await confirmation by studies of patients of other races and HCV-1a. Further prospective studies of a larger number of patients matched for race and HCV genotype are required to explore the relationship between core aa 70, *IL28B* genotype, and hepatocarcinogenesis.

References

1. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, et al. Progress of chronic hepatitis C: results of a large, prospective cohort study. HEPATOLOGY 1998;28:1687-1695.

2. Kenny-Walsh E. Clinical outcomes after hepatitis C infection from contaminated anti-D immune globulin. Irish Hepatology Research Group. N Engl J Med 1999;340:1228-1233.
3. Tsubota A, Arase Y, Someya T, Suzuki Y, Suzuki F, Saitoh S, et al. Early viral kinetics and treatment outcome in combination of high-dose interferon induction vs. pegylated interferon plus ribavirin for naive patients infected with hepatitis C virus of genotype 1b and high viral load. J Med Virol 2005;75:27-34.
4. Koike K. Molecular basis of hepatitis C virus-associated hepatocarcinogenesis: lessons from animal model studies. Clin Gastroenterol Hepatol 2005;3:S132-S135.
5. Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, et al. The core protein of hepatitis C virus induces hepatocellular carcinoma in transgenic mice. Nat Med 1998;4:1065-1067.
6. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372-380.
7. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46:403-410.
8. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol 2007;81:8211-8224.
9. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, 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;52:421-429.
10. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. HEPATOLOGY 2007;46:1357-1364.
11. Fishman SL, Factor SH, Balestrieri C, Fan X, Dibisceglie AM, Desai SM, et al. Mutations in the hepatitis C virus core gene are associated with advanced liver disease and hepatocellular carcinoma. Clin Cancer Res 2009;15:3205-3213.
12. Nakamoto S, Imazeki F, Fukai K, Fujiwara K, Arai M, Kanda T, et al. Association between mutations in the core region of hepatitis C virus genotype 1 and hepatocellular carcinoma development. J Hepatol 2010;52:72-78.
13. Hu Z, Muroyama R, Kowatari N, Chang J, Omata M, Kato N. Characteristic mutations in hepatitis C virus core gene related to the occurrence of hepatocellular carcinoma. Cancer Sci 2009;100:2465-2468.
14. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
15. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
16. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.
17. Rauch A, Kutalik Z, Descombes P, Cai T, di Iulio J, Mueller T, et al., Swiss Hepatitis C and HIV Cohort Studies. Genetic variation in IL28B is associated with chronic hepatitis C and treatment failure — a genome-wide association study. Gastroenterology 2010;138:1338-1345.
18. Fabris C, Falleti E, Cussigh A, Bitetto D, Fontanini E, Bignulin S, et al. IL-28B rs12979860 C/T allele distribution in patients with liver cirrhosis: role in the course of chronic viral hepatitis and the development of HCC. J Hepatol 2011;54:716-722.
19. Bochud P, Bibert S, Kutalik Z, Patin E, Guergnon J, Nalpas B, et al. IL28B alleles associated with poor HCV clearance protect against inflammation and fibrosis in patients infected with non-1 HCV genotypes. HEPATOLOGY 2012;55:384-394.
20. Joshita S, Umemura T, Katsuyama Y, Ichikawa Y, Kimura T, Morita S, et al. Association of IL28B gene polymorphism with development of hepatocellular carcinoma in Japanese patients with chronic hepatitis C virus infection. Hum Immunol 2012;73:298-300.
21. Miura M, Maekawa S, Kadokura M, Sueki R, Komase K, Shindo H, et al. Analysis of viral amino acids sequences and the IL28B SNP influencing the development of hepatocellular carcinoma in chronic hepatitis C. Hepatol Int 2011 Aug 17 [Epub ahead of print].
22. Abe H, Ochi H, Maekawa T, Hayes CN, Tsuge M, Miki D, et al. Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. J Hepatol 2010;53:439-443.
23. Kobayashi M, Suzuki F, Akuta N, Sezaki H, Suzuki Y, Hosaka T, et al. Association of two polymorphisms of the IL28B gene with viral factors and treatment response in 1,518 patients infected with hepatitis C virus. J Gastroenterol 2012;47:596-605.
24. Chayama K, Tsubota A, Arase Y, Saitoh S, Koida I, Ikeda K, et al. Genotypic subtyping of hepatitis C virus. J Gastroenterol Hepatol 1993;8:150-156.
25. Kato N, Hijikata M, Ootsuyama Y, Nakagawa M, Ohkoshi S, Sugimura T, et al. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc Natl Acad Sci U S A 1990;87:9524-9528.
26. Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 2001;46:471-477.
27. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginie deminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395-402.
28. Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, et al. Proteasome activator PA28γ-dependent nuclear retention and degradation of hepatitis C virus core protein. J Virol 2003;77:10237-10249.
29. Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, et al. Critical role of PA28γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis. Proc Natl Acad Sci U S A 2007;104:1661-1666.
30. Funaoka Y, Sakamoto N, Suda G, Itsui Y, Nakagawa M, Kakinuma S, et al. Analysis of interferon signaling by infectious hepatitis C virus clones with substitutions of core amino acids 70 and 91. J Virol 2011;85:5986-5994.
31. Bruno S, Crosignani A, Maisonneuve P, Rossi S, Silini E, Mondelli MU. Hepatitis C virus genotype 1b as a major risk factor associated with hepatocellular carcinoma in patients with cirrhosis: a seventeen-year prospective cohort study. HEPATOLOGY 2007;46:1350-1356.
32. Ikeda K, Saitoh S, Arase Y, Chayama K, Suzuki Y, Kobayashi M, et al. Effect of interferon therapy on hepatocellular carcinogenesis in patients with chronic hepatitis type C: a long-term observation study of 1643 patients using statistical bias correction with proportional hazard analysis. HEPATOLOGY 1999;29:1124-1130.
33. Tarao K, Fujiyama S, Ohkawa S, Miyakawa K, Tamai S, Hirokawa S, et al. Ursodiol use is possibly associated with lower incidence of hepatocellular carcinoma in hepatitis C virus-associated liver cirrhosis. Cancer Epidemiol Biomarkers Prev 2005;14:164-169.
34. Kawaguchi T, Sata M. Importance of hepatitis C virus-associated insulin resistance: therapeutic strategies for insulin sensitization. World J Gastroenterol 2010;16:1943-1952.
35. Kawaguchi T, Taniguchi E, Itou M, Sumie S, Yamagishi S, Sata M. The pathogenesis, complications and therapeutic strategy for hepatitis C virus-associated insulin resistance in the era of anti-viral treatment. Rev Recent Clin Trials 2010;5:147-157.
36. Kawaguchi T, Izumi N, Charlton MR, Sata M. Branched-chain amino acids as pharmacological nutrients in chronic liver disease. HEPATOLOGY 2011;54:1063-1070.
37. Sumida Y, Kanemasa K, Hara T, Inada Y, Sakai K, Imai S, et al. Impact of amino acid substitutions in the hepatitis C virus genotype 1b core region on liver steatosis and glucose tolerance in non-cirrhotic patients without overt diabetes. J Gastroenterol Hepatol 2011;26:836-842.

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Long-term efficacy of interferon therapy in patients with chronic hepatitis B virus infection in Japan

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Abstract
Background Few studies have investigated the long-term effects of interferon (IFN) therapy for chronic hepatitis B (CHB). In this retrospective study, we investigated the efficacy of and predictors of response to IFN therapy in CHB patients.
Methods We analyzed data for 615 Japanese CHB patients (hepatitis B e antigen [HBeAg]-positive 414, HBeAg-negative 201) treated with IFN, and conducted follow up for a median duration of 8.1 years (range 0.5–23.2). Responders were defined as patients who showed continuously normalized alanine transaminase (ALT) levels, HBeAg clearance, and low hepatitis B virus (HBV) DNA levels at 6 months post-treatment or for a span of more than 6 months until each test point at 1, 3, 5, and 10 years.
Results The IFN response rates of all patients were 21, 18, 21, 23, and 25% at 6 months and 1, 3, 5, and 10 years, respectively. On multivariate analysis, significant determinants of the outcome of IFN therapy were as follows: at 6 months and 1 year, young age, low HBV DNA levels, and long duration of treatment; at 3 years, long duration of treatment, young age, and high level of albumin; at 5 years, high level of albumin, female, and pretreated with IFN; and at 10 years, HBeAg-negative. Sixty-nine of the 615 patients (11%) achieved seroclearance of hepatitis B surface antigen (HBsAg). On multivariate analysis, age ≥30 years, HBV genotype A, and male were all independent factors predicting the achievement of HBsAg seroclearance.
Conclusion HBeAg, HBV DNA level, age, sex, albumin, duration of treatment, pretreatment with IFN, and HBV genotype were important factors in determining long-term response to IFN therapy.

Keywords Interferon · Hepatitis B virus · Chronic hepatitis B · Genotype · Hepatitis B surface antigen

Abbreviations

CHB	Chronic hepatitis B
HBV	Hepatitis B virus
IFN	Interferon
HBeAg	Hepatitis B e antigen
ALT	Alanine transaminase
MU	Million units
HBsAg	Hepatitis B surface antigen
CLEIA	Chemiluminescent enzyme immunoassay
bDNA	Branched-chain DNA probe assay
TMA-HPA	Transcription-mediated amplification and hybridization protection assay
PCR	Polymerase chain reaction
ELISA	Enzyme-linked immunosorbent assay
AST	Aspartate transaminase
AFP	α Fetoprotein
OR	Odds ratio
CI	Confidence interval
HCC	Hepatocellular carcinoma

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Introduction

Hepatitis B virus (HBV) infection is a common disease that can induce a chronic carrier state and is associated with the risk of developing progressive disease and hepatocellular carcinoma [1]. Interferon (IFN) and several nucleoside/nucleotide analogues such as lamivudine, adefovir dipivoxil, entecavir, and tenofovir disoproxil fumarate are currently approved as treatments for chronic hepatitis B (CHB) in most countries [2–5]. Successful treatment of CHB with clearance of hepatitis B e antigen (HBeAg), reduction in serum HBV DNA levels, and normalization of alanine transaminase (ALT) levels is associated with a favorable long-term outcome, independent of the antiviral drug used [6, 7].

A meta-analysis of IFN therapy published in 1993 reviewed 15 randomized controlled studies involving 837 adult patients who received IFN-α at doses of 5–10 million units (MU) administered at intervals ranging from daily to three times weekly for 4–6 months [8]. Clearance of HBeAg was noted in 33% of the treated patients compared with 12% of controls. Elimination of detectable HBV DNA and normalization of ALT levels were also more common in the treated patients than in the controls. The major pretreatment factors that correlated with a response were high ALT levels [9–11], low HBV DNA levels [9, 10], female sex, and elevated liver activity and fibrosis on liver biopsy [8]. Another recent meta-analysis of 24 randomized controlled trials concluded that the rates of persistent ALT normalization, clearance of HBeAg, and sustained elimination of HBV DNA (determined by hybridization) induced by IFN therapy were approximately 25% greater than the rates for controls. A more recent meta-analysis report showed that IFN increased the incidence of HBeAg and hepatitis B surface antigen (HBsAg) seroclearance after long-term follow up of 3–7 years [12].

However, specific data on the long-term effects of IFN therapy (median follow-up duration of 8.1 years), particularly among the Japanese, are limited. Moreover, few reports have investigated factors predicting the achievement of HBsAg seroclearance. To further evaluate factors influencing clinical outcome, we performed a retrospective cohort study on CHB patients treated with IFN in our hospital.

Patients and methods

Patients

We retrospectively examined 615 Japanese patients (151 females and 464 males) who commenced IFN treatment between June 1984 and April 2008 in the Department of Hepatology at Toranomon Hospital (Table 1). Several of the patients have been included in previous reports [13–15].

Table 1 Characteristics of patients at commencement of interferon therapy

Demographic data	
Total number	615
Sex, female/male	151/464
Age, years (range)	35 (15–68)
Previously treated with interferon	123 (20%)
Duration of treatment, weeks (range)	26 (4–981)
Follow-up period, years (range)	8.1 (0.5–23.2)
Laboratory data	
Aspartate transaminase, IU/L (range)	72 (18–990)
Alanine transaminase, IU/L (range)	138 (12–1578)
Bilirubin, mg/dL (range)	0.7 (0.2–8.8)
Albumin, g/dL (range)	3.9 (2.6–5.3)
Platelets, $\times 10^3/\mu L$ (range)	174 (48–500)
Staging of liver histology (F0/1/2/3/4/ND)	8/77/185/162/72/111
Serum HBV DNA, log copies/mL (range)	>7.6 (<2.6 to >7.6)
HBeAg (positive/negative)	414/201
HBV genotype (A/B/C/D/H/B + C/unknown)	24/37/504/1/1/1/47

Values are expressed as medians and ranges (in parentheses) or as numbers and percentages (in parentheses)

HBV hepatitis B virus, *HBeAg* hepatitis B e antigen, *ND* not done

All enrolled patients were followed up for a range of 0.5–23.2 years from completion of IFN treatment, with a median follow-up duration of 8.1 years. Before the commencement of IFN treatment, all patients had been positive for HBsAg in the serum for more than 6 months, and all were confirmed to have hepatitis caused by HBV and not by another vector, such as infection with hepatitis C virus or autoimmune hepatitis. None had a history of drug abuse or alcoholic hepatitis, and none had received nucleoside/nucleotide analogue therapy. The study was conducted in accordance with the ethical principles of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethics Committee. Informed consent was obtained from each patient.

Interferon therapy and assessment of response to therapy

Patients received 3–12 MU of IFN-α or IFN-β (Sumiferon: Dainippon Sumitomo Pharma, Osaka, Japan; Canferon A: Takeda Chemical Industries, Osaka, Japan; Intron A: Schering-Plough MSD KK, Osaka, Japan; and Feron: Toray, Tokyo, Japan). The durations and regimens of treatment were as follows: 4 weeks (89 patients; daily for

4 weeks), 26 weeks (270 patients; daily for 4 weeks followed by 2 or 3 times a week), 52 weeks (103 patients; 2 or 3 times a week), 104 weeks (80 patients; 2 or 3 times a week), and more than 104 weeks (73 patients; 2 or 3 times a week). The median duration of treatment was 26 weeks (range 4–981).

The numbers of responders were evaluated at 6 months and 1, 3, 5, and 10 years after the completion of IFN therapy. In the baseline HBeAg-positive patients, responders were defined as patients who showed normalization of serum ALT level (normal level 6–30 IU/L), HBeAg clearance, and low HBV DNA level (<5 log copies/mL) at 6 months after completion of IFN therapy. In addition, baseline HBeAg-positive patients who showed continuous normalization of ALT levels, HBeAg clearance, and low HBV DNA level for more than 6 months until each test point at 1, 3, 5, and 10 years after completion of IFN therapy were also classified as "responders." In the baseline HBeAg-negative patients, responders were defined as those who showed sustained normalization of ALT level and low HBV DNA level (<4 log copies/mL) for more than 6 months until each test point after completion of IFN therapy.

All patients not considered to be responders were termed "non-responders." Patients receiving other therapies (IFN or nucleoside/nucleotide analogues) after the completion of IFN therapy were also termed non-responders.

Blood tests and serum viral markers

Routine biochemical tests were performed monthly via standard procedures during and for the first 12 months following the completion of IFN treatment and at least every 2 months thereafter. Levels of HBsAg, HBeAg, and anti-HBe were determined using radioimmunoassay kits (Abbott Diagnostics, Chicago, IL, USA) or a chemiluminescent enzyme immunoassay (CLEIA; Lumipulse System; Fujirebio, Tokyo, Japan). HBV DNA levels were measured using a branched-chain DNA probe assay (bDNA) (Chiron Laboratory Service, Van Nuys, CA, USA), a transcription-mediated amplification and hybridization protection assay (TMA-HPA) (Chugai Diagnostics Science, Tokyo, Japan), or a polymerase chain reaction (PCR)-based assay (COBAS Amplicor HBV Monitor Test or COBAS TaqMan HBV Test; Roche Diagnostics, Indianapolis, IN, USA).

HBV genotype

The major genotypes of HBV were determined using an enzyme-linked immunosorbent assay (ELISA; Institute of Immunology, Tokyo, Japan) or a PCR-invader assay (BML, Tokyo, Japan) according to the methods described by Usuda et al. [16] or Tadokoro et al. [17].

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 or Fisher's exact test and Mann–Whitney U-test where appropriate. Independent predictive factors associated with response to IFN treatment were determined using multivariate multiple logistic regression. The following 14 potential predictors of response to IFN treatment were assessed in this study: age, sex, pretreatment with IFN, duration of IFN treatment, severity of liver disease (CH or liver cirrhosis), HBV genotype, and levels of aspartate transaminase (AST), ALT, bilirubin, albumin, platelets, α fetoprotein (AFP), HBeAg, and HBV DNA. All factors found to be at least marginally associated with response to IFN therapy ($P < 0.10$) were entered into the multivariate multiple logistic regression analysis. The above calculations were performed using the Windows SPSS software package version 11.0.1 J (SPSS, Chicago, IL, USA).

The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative risk. Independent risk factors predicting the achievement of HBsAg seroclearance were studied using stepwise Cox regression analysis. Potential factors predicting the achievement of HBsAg seroclearance assessed here were the above 14 variables, each transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBsAg seroclearance ($P < 0.10$) were tested in the multivariate Cox proportional hazard model. A Kaplan–Meier estimate was performed using the SPSS software, and P values were calculated using the Cox-Mantel log-rank test. A two-tailed P value of <0.05 was considered statistically significant.

Results

Study population

Twenty-four (4%), 37 (6%), 504 (82%), 1 (0.2%), 1 (0.2%), and 1 (0.2%) patients were infected with HBV genotypes A, B, C, D, H, and B + C, respectively. Genotype could not be measured in the remaining 47 patients. The baseline characteristics of the patients are shown in Table 1. Although few patients had genotypes A and B, the distribution of HBV genotype was similar to that in patients with CHB who had received care in our hospital, with a follow-up period of more than 2 years [18]. Twenty-two of 24 patients with genotype A, 14 of 37 with

genotype B, 342 of 504 with genotype C, 1 of 1 with genotype H, and 34 of 47 with unknown genotype were HBeAg-positive at the commencement of treatment. While we were able to measure HBV DNA levels in 254 patients at the commencement of IFN therapy, levels in the remaining 361 could not be measured owing to a lack of commercial kits before the bDNA assay was available. The numbers of patients receiving other additional therapies after the completion of IFN therapy were 111 (HBeAg-positive/-negative, 90/21), 92 (67/25), 34 (25/9), and 61 (39/22) at the 1-, 3-, 5-, and 10-year time points, respectively.

Response to interferon therapy in all patients

The IFN response rates in all patients were 21% (105/497), 18% (86/491), 21% (90/428), 23% (82/359), and 25% (59/235) at 6 months and 1, 3, 5, and 10 years, respectively, after completion of the IFN therapy (Fig. 1). In patients with genotype A, the response rate was highest at 6 months post-treatment and gradually decreased at subsequent time points from 1 to 10 years thence. In patients with genotype B, response rates were over 20% at all time points except for 6 months post-treatment, whereas rates in patients with genotype C were under 25% at all time points (Fig. 2a).

Evaluation of efficacy of IFN in relation to clinical factors in all patients

The data of all patients were subjected to univariate analyses to determine the clinical factors contributing to the efficacy of IFN at each time point. We then investigated the significance of response to IFN therapy using multivariate logistic regression analysis.

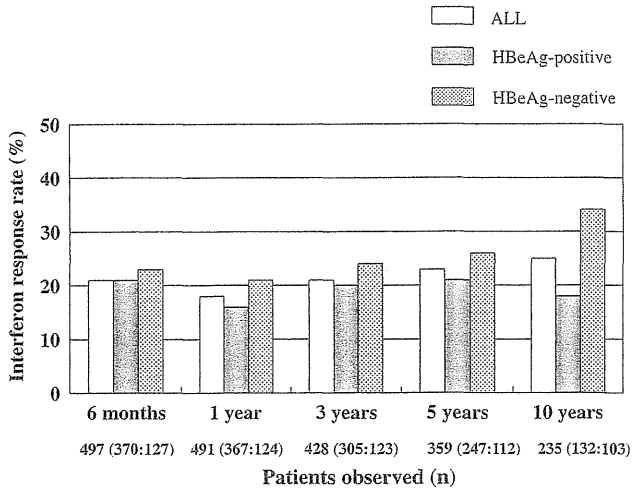

Fig. 1 Interferon response rates of all patients and hepatitis B e antigen (*HBeAg*)-positive and HBeAg-negative patients at 6 months and 1, 3, 5, and 10 years

Multivariate analyses including the variables noted above revealed several parameters that independently influenced the outcome of IFN therapy; namely, at 6 months: age ($P = 0.013$), HBV DNA level ($P = 0.019$), and duration of treatment ($P = 0.034$); at 1 year: HBV DNA level ($P < 0.001$) and age ($P = 0.001$); at 3 years: duration of treatment ($P < 0.001$), age ($P = 0.013$) and albumin level ($P = 0.013$); at 5 years: albumin level ($P = 0.004$), sex ($P = 0.005$), and pretreatment with IFN ($P = 0.039$); and at 10 years: HBeAg ($P < 0.001$) (Table 2).

Response to interferon therapy and evaluation of efficacy of IFN in relation to clinical factors in HBeAg-positive patients

Response rates in baseline HBeAg-positive patients were 21% (76/370), 16% (60/367), 20% (61/305), 21% (53/247), and 18% (24/132) at 6 months and 1, 3, 5, and 10 years, respectively (Fig. 1). In patients with genotype A, the response rate was highest at 6 months post-treatment and the rate was roughly equivalent to the 6 months post-treatment rate at subsequent time points from 1 to 10 years. Response rates in patients with genotype B in particular were above 40% at all time points except at 6 months, although few patients had genotype B. On the other hand, response rates in patients with genotype C were under 20% at all time points (Fig. 2a).

In addition, multivariate analyses in HBeAg-positive patients also revealed several parameters that independently influenced the outcome of IFN therapy–at 6 months: duration of treatment ($P = 0.001$) and age ($P = 0.014$); at 1 year: age ($P = 0.011$) and HBV DNA level ($P = 0.027$); at 3 years: sex ($P = 0.008$), duration of treatment ($P = 0.019$), age ($P = 0.020$), pretreatment with IFN ($P = 0.029$), and albumin level ($P = 0.043$); at 5 years: sex ($P = 0.002$) and pretreatment with IFN ($P = 0.005$); and at 10 years, genotype ($P = 0.019$) and AST ($P = 0.035$) (Table 3).

Response to interferon therapy and evaluation of efficacy of IFN in relation to clinical factors in HBeAg-negative patients

Response rates in baseline HBeAg-negative patients were 23% (29/127), 21% (26/124), 24% (29/123), 26% (29/112), and 34% (35/103) at 6 months and 1, 3, 5, and 10 years, respectively (Fig. 1). Rates in patients with genotype C were gradually increased at subsequent time points, whereas those in patients with genotype B remained under 30% at all time points (Fig. 2b).

In addition, univariate and multivariate analyses in HBeAg-negative patients revealed that duration of treatment (≥1 year) independently influenced the outcome of

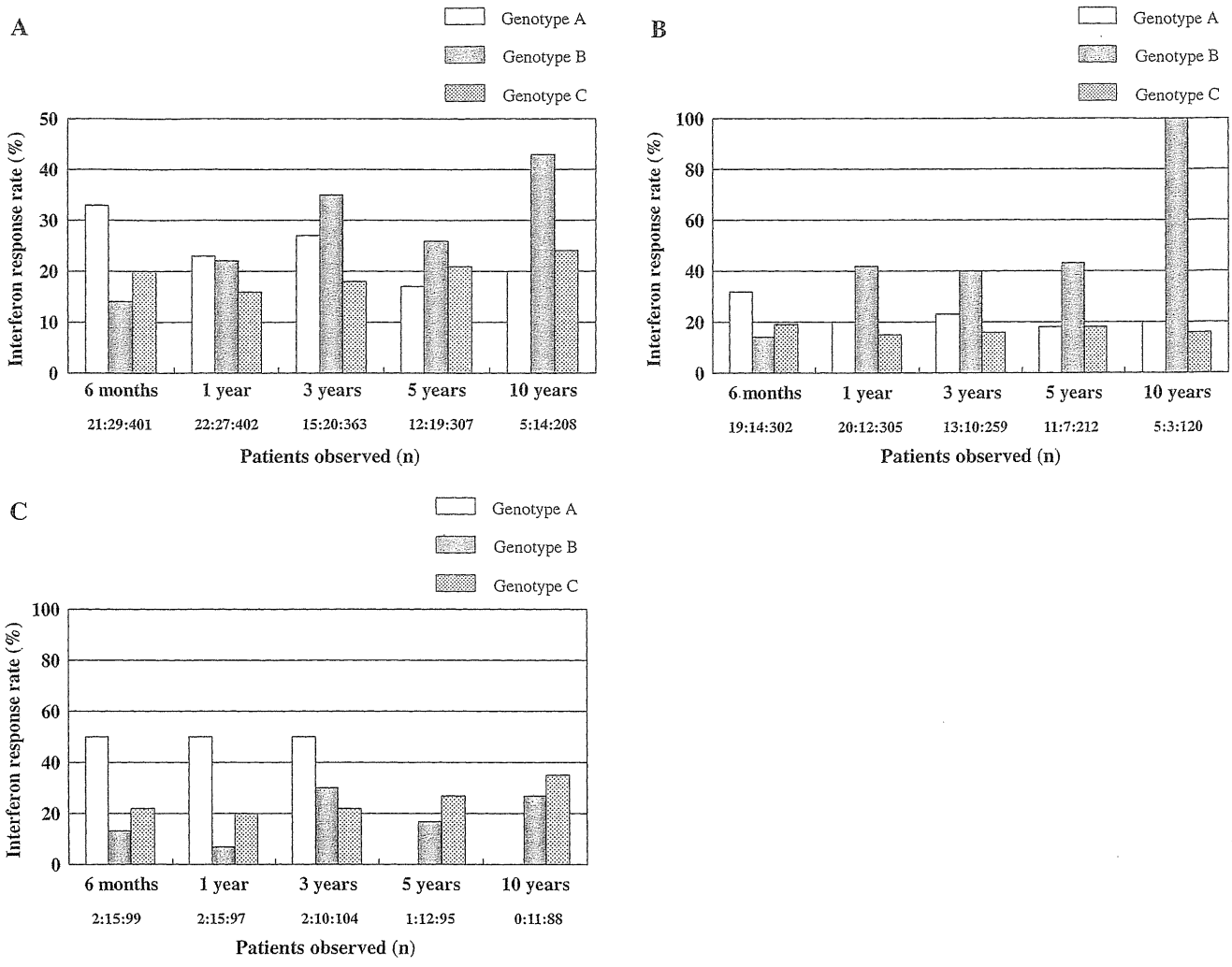

Fig. 2 Interferon response rates of patients with genotypes A, B, and C at 6 months and 1, 3, 5, and 10 years. **a** All patients, **b** HBeAg-positive patients, **c** HBeAg-negative patients

IFN therapy at 6 months, and at 1 and 3 years. No parameters independently influenced the outcome of IFN therapy at 5 or 10 years.

Evaluation of efficacy of IFN in relation to HBs antigen seroclearance

The HBsAg seroclearance rate in this study was obtained from patients who received IFN therapy alone; 69 of 615 patients (11%) achieved seroclearance of HBsAg. The cumulative HBsAg seroclearance rates in all patients from the commencement date of IFN therapy were 6.5% at 5 years, 15% at 10 years, 35% at 15 years, and 44% at 20 years (Kaplan–Meier method; Fig. 3a). No patients experienced the reappearance of HBsAg after seroclearance. Five factors found to be associated with achievement of HBsAg seroclearance on univariate analysis were: male sex ($P = 0.002$), age $\geq$30 years ($P = 0.011$), genotype A ($P = 0.038$), HBeAg-negativity ($P = 0.045$), and bilirubin $\leq$1.0 mg/dL ($P = 0.064$). On multivariate analysis, independent factors predicting the achievement of HBsAg seroclearance were: age $\geq$30 years, genotype A, and male sex (Table 4). The cumulative HBsAg seroclearance rate for genotype A patients was significantly higher than the rate for those with genotypes B or C ($P = 0.0116$) (Fig. 3b).

Relationship between the response to IFN and the development of hepatocellular carcinoma

Twenty-nine patients developed hepatocellular carcinoma (HCC) during the observation period, excluding 17 patients who received other additional therapies after the completion of IFN therapy and developed HCC thereafter. IFN response rates in the 29 patients who developed HCC were 5% (1/22), 5% (1/20), 10% (2/20), 13% (2/15), and 13% (2/16), respectively, at 6 months and 1, 3, 5, and 10 years after the completion of IFN. No patient developed HCC after HBsAg seroclearance.

Table 2 Factors associated with response to interferon therapy for all patients at 6 months and 1, 3, 5, and 10 years

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
6 Months after completion of IFN therapy ($n = 229$)				
Duration of treatment (≥1 year)	2.680 (1.724–4.166)	<0.001	2.107 (1.058–4.198)	0.034
HBV DNA level (≤7.0 log copies/mL)	2.165 (1.107–4.219)	0.026	2.309 (1.148–4.630)	0.019
Age (<30 years)		0.057	2.451 (1.209–4.950)	0.013
1 year after completion of IFN therapy ($n = 231$)				
Duration of treatment (≥1 year)	2.553 (1.588–4.104)	<0.001		
HBV DNA level (≤7.0 log copies/mL)	3.268 (1.597–6.667)	0.001	4.464 (2.058–9.709)	<0.001
Age (<35 years)	1.799 (1.125–2.874)	0.014	3.831 (1.718–8.547)	0.001
3 years after completion of IFN therapy ($n = 397$)				
Duration of treatment (≥1 year)	2.410 (1.495–3.885)	<0.001	2.739 (1.618–4.634)	<0.001
Age (<30 years)	2.070 (1.215–3.521)	0.009	2.110 (1.171–3.802)	0.013
Albumin (≥3.9 g/dL)	1.697 (1.045–2.757)	0.030	2.009 (1.158–3.486)	0.013
Genotype (non-C)	2.155 (1.033–4.504)	0.041		
5 years after completion of IFN therapy ($n = 356$)				
Albumin (≥3.9 g/dL)	1.869 (1.108–3.153)	0.017	2.321 (1.316–4.093)	0.004
Pretreatment with IFN (positive)	1.770 (1.016–3.084)	0.048	1.821 (1.029–3.222)	0.039
Sex (female)		0.060	2.381 (1.297–4.367)	0.005
Duration of treatment (≥1 year)		0.080		
10 years after completion of IFN therapy ($n = 234$)				
HBeAg (negative)	2.315 (1.269–4.219)	0.006	2.252 (1.230–4.115)	0.009
ALT (≥100 IU/L)	1.972 (1.053–3.690)	0.036		
Pretreatment with IFN (positive)		0.058		

ALT alanine transaminase, *IFN* interferon, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *CI* confidence interval, *OR* odds ratio, *n* number submitted to multivariate analysis, including all factors found to be associated with response to IFN therapy

Discussion

Although IFN has been reported to exert beneficial effects in CHB patients, the response rate is not high. A meta-analysis published in 1993 reviewed 15 randomized controlled studies involving 837 adult patients who received IFN-α for 4–6 months, and elimination of HBeAg occurred in 33% of the treated patients [8]. In previous studies, we found the response rates among HBeAg-positive patients at 6 months after the completion of therapy to be 20 and 31% for 6 months and 1 year of IFN therapy, respectively [13, 15]. Although a recent meta-analysis reported that IFN increased the incidence of HBeAg and HBsAg seroclearance after long-term follow up of 3–7 years [12], the factors that influenced the clinical outcome were unclear.

In Japan, from 1988, 4-week IFN treatment was reimbursed by the healthcare system, and since 2002, 24-week IFN treatment has been conducted. In the present study, these two regimens were the major ones, and other regimens were used in clinical studies at our hospital (including previously reported studies [14, 15]). Although the durations of treatment differed, we analyzed the factors associated with long-term response to IFN therapy, including the factor of duration of treatment.

In the present study, response rates were low among HBeAg-positive patients and relatively high among HBeAg-negative patients at all time points. Approximately 20% of the HBeAg-positive patients had sustained a response at 6 months to 10 years of follow up. Long-term follow-up studies after a four- to six-month course of IFN therapy in HBeAg-positive patients in European and Taiwanese studies showed higher (33–75%) response rates (HBeAg loss) than our study [7, 19, 20]. The difference in response rates between our present study and previous studies in other countries may be due to differences in ethnicity or HBV genotype (mainly genotype C in Japan). Moreover, the low IFN response rates at 1, 3, 5, and 10 years in the HBeAg-positive patients in our study were likely due to the change in treatments (IFN or nucleoside/nucleotide analogues). On the other hand, the response rates of HBeAg-negative patients in the present study were about 20% at 6 months and gradually increased thereafter. The sustained response rate in HBeAg-negative patients was usually <30% in European studies [21–23]. The response

Table 3 Factors associated with response to interferon therapy for HBeAg-positive patients at 6 months and 1, 3, 5, and 10 years

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*P*	OR (95% CI)	*P*
6 months after completion of IFN therapy ($n = 279$)				
Duration of treatment (≥1 year)	2.449 (1.457–4.114)	0.001	2.801 (1.540–5.096)	0.001
Age (<35 years)	1.855 (1.112–3.096)	0.017	2.128 (1.164–3.891)	0.014
1 year after completion of IFN therapy ($n = 172$)				
Duration of treatment (≥1 year)	2.483 (1.407–4.380)	0.002		
HBV DNA level (≤7.0 log copies/mL)	3.509 (1.495–8.264)	0.005	3.003 (1.130–7.937)	0.027
Age (<35 years)	1.996 (1.133–3.521)	0.015	3.610 (1.351–9.615)	0.011
3 years after completion of IFN therapy ($n = 283$)				
Age (<35 years)	2.041 (1.155–3.597)	0.013	2.083 (1.122–3.861)	0.020
Duration of treatment (≥1 year)	2.055 (1.153–3.661)	0.016	2.130 (1.132–4.008)	0.019
Pretreatment with IFN (positive)	2.054 (1.050–4.019)	0.041	2.336 (1.091–4.998)	0.029
Albumin (≥3.9 g/dL)		0.055	1.974 (1.020–3.820)	0.043
Sex (female)		0.089	2.646 (1.284–5.464)	0.008
5 years after completion of IFN therapy ($n = 247$)				
Sex (female)	2.571 (1.328–4.975)	0.006	2.924 (1.477–5.814)	0.002
Pretreatment with IFN (positive)	2.460 (1.213–4.988)	0.015	2.870 (1.377–5.980)	0.005
10 years after completion of IFN therapy ($n = 122$)				
Genotype (non-C)	5.319 (1.222–23.26)	0.032	6.410 (1.364–30.30)	0.019
AST (≥100 IU/L)		0.081	2.932 (1.078–7.972)	0.035

AST aspartate transaminase, *IFN* interferon, *HBV* hepatitis B virus, *HBeAg* hepatitis B e antigen, *CI* confidence interval, *OR* odds ratio, *n* number submitted to multivariate analysis, including all factors found to be associated with response to IFN therapy

rates of HBeAg-negative patients in our present study and the studies in other countries [21–23] were similar.

Few reports have identified the factors associated with long-term virological response to IFN therapy. In our present study, HBeAg-negativity was the most important factor for predicting a long-term response (10 years). While the HBV DNA level was important for predicting the response at 6 months and 1 year for all patients and the response at 1 year for HBeAg-positive patients, other factors (age, sex, albumin level, AST, IFN pretreatment, and duration of treatment) were found to be important at some time points for all patients and for HBeAg-positive patients. The HBV DNA level may not have been associated with long-term response to IFN therapy because the follow-up period (median 5.7 years) in patients with an HBV DNA level measurable with commercial kits was significantly shorter than that in the other patients (median 11.2 years; $P < 0.001$).

Previous studies have reported that high ALT levels, low HBV DNA level, female sex, and elevated liver activity and level of fibrosis on liver biopsy were major pretreatment factors correlated with a response to IFN [8–11, 24]. However, in these studies the follow-up times for judging the response were short (typically 6 months to 1 year). Our present study has clarified that HBeAg, HBV DNA level, age, sex, IFN pretreatment, duration of treatment, and levels of albumin and AST are important factors in the long-term response to IFN. Further, non-C genotype was an important factor for long-term response in HBeAg-positive patients. Kao et al. [25] and Lin et al. [20] reported that HBV genotype B was associated with a higher response rate to IFN-α therapy than genotype C among CHB patients positive for HBeAg. Similarly, response rates among HBeAg-positive patients with genotype B in the present study were also higher than the response rates in those with genotype C in terms of long-term response (Fig. 2b). The long-term response rate among HBeAg-negative patients was relatively higher than that in HBeAg-positive patients. Previous reports have shown that response rates to a 6- to 12-month course of IFN-α in HBeAg-negative CHB patients range from 10 to 47% (average 24%) [26–29]. In addition, our previous report showed that 9 of 12 (75%) patients who received IFN-β twice per week for 24 weeks responded to the therapy [14]. However, the follow-up periods of these studies were short, and the long-term efficacy has not been clarified. While the efficacy of IFN in HBeAg-negative patients was high in the present study, the factors that might be useful in predicting a sustained response were less well-defined than those in HBeAg-positive patients, as previously reported [5].

A meta-analysis of IFN therapy published in 2010 reviewed 6 clinical controlled studies including 828 patients who received IFN [12]. The duration of follow-up

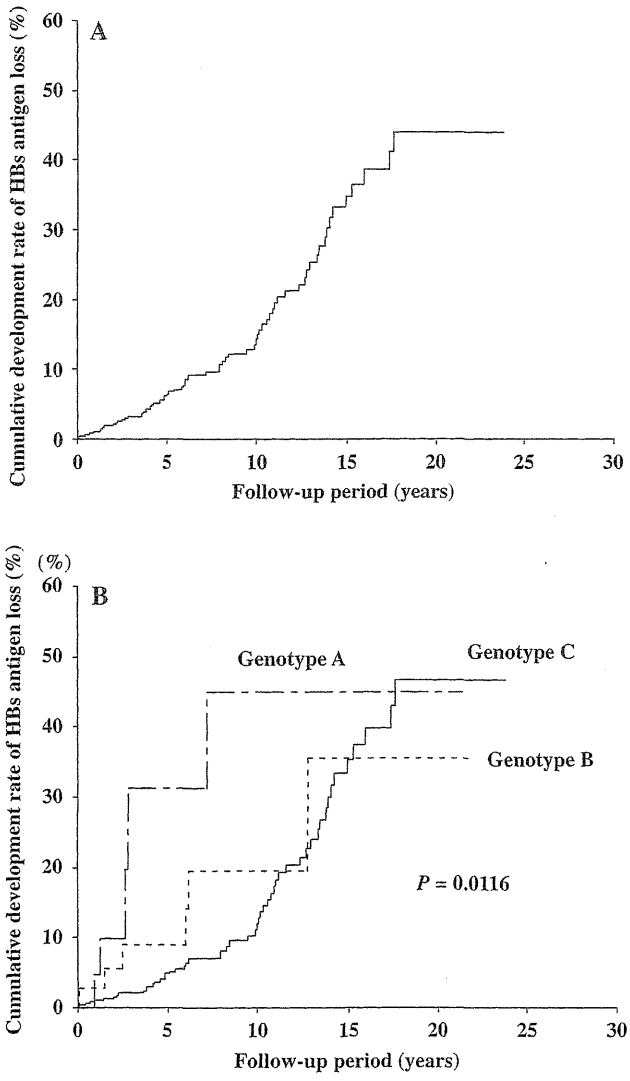

Fig. 3 Cumulative clearance of hepatitis B surface (*HBs*) antigen in patients treated with interferon (Kaplan–Meier method). **a** All patients, **b** patients stratified by genotypes A, B, and C

Table 4 Factors associated with HBsAg seroclearance by interferon therapy, determined by multivariate analysis

Parameter	Category	Hazard ratio	95% CI	*P*
Age	<30 years	1		0.002
	≥30 years	4.433	1.703–11.538	
Genotype	A	1		0.004
	B	0.296	0.087–1.005	
	C	0.199	0.075–0.528	
Sex	Female	1		0.005
	Male	2.962	1.387–6.327	

HBsAg hepatitis B surface antigen, *CI* confidence interval

ranged from 35.8 months to 7 years, and HBsAg seroclearance occurred in 9.5% (79/828). In the present study, we observed HBsAg seroclearance in 69 of 615 (11%) patients, with a median follow-up duration of 8.1 years. However, few reports have investigated factors predicting the achievement of HBsAg seroclearance. In our study, important factors for achieving HBsAg seroclearance were age ≥30 years, genotype A, and male sex. Patients with genotype A had primarily been infected during adulthood via sexual contact, and the average duration of infection was relatively short. In contrast, most Japanese carriers are infected perinatally and possess HBV genotype C, and therefore the efficacy of IFN therapy for patients with genotype C may be low. Male sex was also an important factor in determining potential to achieve HBsAg seroclearance, although female sex was an important factor in determining long-term response to IFN therapy. In our previous study of HBsAg seroclearance (mainly spontaneous seroclearance), we found that response rates were low among females (19%; 45/231) [30]. These present and previous findings indicate that male patients tended to achieve HBsAg seroclearance more frequently than females, although the reason is unclear. We previously reported that Kaplan–Meier analysis in 486 patients who received lamivudine therapy for 5 and 10 years showed an estimated loss of HBsAg in 3 and 13% of the patients, respectively, [31]. The cumulative clearance rates of HBsAg, also determined by Kaplan–Meier analysis, in patients treated with IFN were higher than those in the patients treated with lamivudine, albeit that there were differences in the baseline characteristics of the patients at the commencement of the respective therapies. The effects of IFN therapy in modulating the host immune response might induce HBsAg clearance.

In conclusion, we investigated the long-term efficacy of IFN therapy in Japanese CHB patients. Response rates were low among HBeAg-positive patients and relatively high among HBeAg-negative patients at all time points examined. HBeAg-negative status, HBV DNA level, age, sex, pretreatment with IFN, duration of treatment, and levels of albumin and AST were important factors in predicting long-term response for all patients and for HBeAg-positive patients. Age, genotype, and sex were important factors in predicting ability to achieve HBsAg seroclearance. Further studies exploring the efficacy of therapy over a longer duration may be necessary to confirm these findings and establish true response rates to IFN therapy, including treatment with pegylated IFN.

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References

1. Beasley RP, Hwang LW, Lin CC, Chien CS. Hepatocellular carcinoma and hepatitis B virus. A prospective study of 22,707 men in Taiwan. Lancet. 1981;2:1129–233.
2. Dienstag JL, Perrillo RP, Schiff ER, Bartholomew M, Vicary C, Rubin M. A preliminary trial of lamivudine for chronic hepatitis B infection. N Engl J Med. 1995;333:1657–61.
3. Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, et al. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. N Engl J Med. 2003; 348:808–16.
4. Chang TT, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, et al. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. N Engl J Med. 2006;354:1001–10.
5. Lok ASF, Heathcote EJ, Hoofnagel JH. Management of hepatitis B: 2000–summary of a workshop. Gastroenterology. 2001;120: 1828–53.
6. Liaw YF, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, et al. Lamivudine for patients with chronic hepatitis B and advanced liver disease. N Engl J Med. 2004;351:1521–31.
7. van Zonneveld M, Honkoop P, Hansen BE, Niesters HG, Darwish Murad S, de Man RA, et al. Long-term follow-up of alpha-interferon treatment of patients with chronic hepatitis B. Hepatology. 2004;39:804–10.
8. Wong DHK, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. Ann Intern Med. 1993;119:312–23.
9. Brook MG, Karayiannis P, Thomas HC. Which patients with chronic hepatitis B virus infection will respond to alpha-interferon therapy? Hepatology. 1989;10:761–3.
10. Perrillo RP, Schiff ER, Davis GL, Bodenheimer HC Jr, Lindsay K, Payne J, et al. A randomized, controlled trial of interferon alfa-2b alone and after prednisolone withdrawal for the treatment of chronic hepatitis B. N Engl J Med. 1990;323:295–301.
11. Perrillo RP, Lai CL, Liaw YF, Dienstag JL, Schiff ER, Schalm SW, et al. Predictors of HBeAg loss after lamivudine treatment for chronic hepatitis B. Hepatology. 2002;36:186–94.
12. Yang YF, Zhao W, Xia HM, Zhong YD, Huang P, Wen J. Long-term efficacy of interferon alpha therapy on hepatitis B viral replication in patients with chronic hepatitis B: a meta-analysis. Antiviral Res. 2010;85:361–5.
13. Suzuki F, Arase Y, Akuta N, Tsubota A, Suzuki Y, Sezaki H, et al. Efficacy of 6-month interferon therapy in chronic hepatitis B virus infection in Japan. J Gastroenterol. 2004;39:969–74.
14. Arase Y, Chayama K, Tsubota A, Murashima N, Suzuki Y, Koida I, et al. A randomized, double-blind, controlled trial of natural interferon-α therapy for e-antigen-negative chronic hepatitis B patients with abnormal transaminase levels. J Gastroenterol. 1996;31:559–64.
15. Arase Y, Tsubota A, Saitoh S, Suzuki Y, Kobayashi M, Suzuki F, et al. Randomized, controlled trial of natural interferon-α therapy for e-antigen-positive chronic hepatitis B patients. Hepatol Res. 2002;23:98–104.
16. Usuda S, Okamoto H, Imawari H, Baba K, Tsuda F, Miyakawa Y, et al. Serological detection of hepatitis B virus genotypes by ELISA with monoclonal antibodies to type-specific epitopes in preS2-region product. J Virol Method. 1999;80:97–112.
17. Tadokoro K, Kobayashi M, Yamaguchi T, Suzuki F, Miyauchi S, Egashira T, et al. Classification of hepatitis B virus genotypes by the PCR-Invader method with genotype-specific probes. J Virol Method. 2006;138:30–9.
18. Kobayashi M, Arase Y, Ikeda K, Tsubota A, Suzuki Y, Saitoh S, et al. Clinical characteristics of patients infected with hepatitis B virus genotypes A, B and C. J Gastroenterol. 2002;37:35–9.
19. Niderau C, Heintges T, Lange S, Goldmann G, Niederau CM, Mohr L, et al. Long-term follow-up of HBeAg-positive patients treated with interferon alfa for chronic hepatitis B. N Engl J M. 1996;334:1422–7.
20. Lin SM, Yu KL, Lee CM, Chien RN, Sheen IS, Chu CM, et al. Interferon therapy in HBeAg positive chronic hepatitis reduces progression to cirrhosis and hepatocellular carcinoma. J Hepatol. 2007;46:45–52.
21. Papatheodoridis GV, Manesis E, Hadziyannis SJ. The long-term outcome of interferon-α treated and untreated patients with HBeAg-negative chronic hepatitis B. J Hepatol. 2001;34:306–13.
22. Brunetto MR, Oliveri F, Coco B, Leandro G, Colombatto P, Gorin JM, et al. Outcome of anti-HBe positive chronic hepatitis B in alpha-interferon treated and untreated patients: a long term cohort study. J Hepatol. 2002;36:263–70.
23. Lampertico P, Ninno ED, Vigano M, Romeo R, Donato MF, Sablon E, et al. Long-term suppression of hepatitis B e antigen-negative chronic hepatitis B by 24-month interferon therapy. Hepatology. 2003;37:756–63.
24. Lau DTY, Everhart J, Kleiner DE, Park Y, Vergalla J, Schmid P, et al. Long-term follow-up of patients with chronic hepatitis B treated with interferon alfa. Gastroenterology. 1997;113:1660–7.
25. Kao JH, Wu NH, Chen PJ, Lai MY, Chen DS. Hepatitis B genotypes and the response to interferon therapy. J Hepatol. 2000;33:998–1002.
26. Hadziyannis S, Bramou T, Makris A, Moussoulis G, Zignego L, Papaioannou C. Interferon alfa-2b treatment of HBeAg negative/serum HBV DNA positive chronic active hepatitis B. J Hepatol. 1990;11:S133–6.
27. Pastore G, Santantonio T, Milella A, Monno L, Mariano N, Moschetta R, et al. Anti-HBe-positive chronic hepatitis B with HBV-DNA in serum: response to a 6-month course of lymphoblastoid interferon. J Hepatol. 1992;20:221–5.
28. Fattovich G, Farci P, Rugge M, Brollo G, Mandas A, Pontisso P, et al. A randomized, controlled trial of lymphoblastoid interferon-alfa in patients with chronic hepatitis B lacking HBeAg. Hepatology. 1992;15:584–9.
29. Lampertico P, Del Ninno E, Manzin A, Donato MF, Rumi MG, Lunghi G, et al. A randomized, controlled trial of a 24-month course of interferon alfa 2b in patients with chronic hepatitis B who had hepatitis B virus DNA without hepatitis B e antigen in serum. Hepatology. 1997;26:1621–5.
30. Arase Y, Ikeda K, Suzuki F, Suzuki Y, Saitoh S, Kobayashi M, et al. Long-term outcome after hepatitis B surface antigen seroclearance in patients with chronic hepatitis B. Am J Med. 2006;119:71 e9–e16.
31. Kobayashi M, Suzuki F, Akuta N, Hosaka T, Sezaki H, Yatsuji H, et al. Loss of hepatitis B surface antigen from the serum of patients with chronic hepatitis treated with lamivudine. J Med Virol. 2007;79:1472–7.

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Short Communication

Prevalence of hepatitis C virus variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients with genotype 1b

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ABSTRACT

Background: Hepatitis C virus (HCV) of genotype 1b is the most prevalent worldwide, and the least responsive to interferon-based treatments. A combination therapy with two direct-acting antivirals has shown promising results in patients with HCV-1b, but the prevalence of drug-resistant variants before treatment is not known in the Japanese population.
Objectives: To detect HCV variants resistant to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052) in hepatitis patients infected with HCV-1b.
Study design: Drug-resistant mutations were determined in the 362 hepatitis patients infected with HCV-1b who had not received direct-acting antivirals before.
Results: Amino-acid substitutions resistant to NS3 inhibitors (V36A, T54S, Q80H and D168E) were detected in 15 of the 307 (4.9%) patients, who had been examined, and T54S (3.3%) predominated over V36A (0.3%), Q80R (0.7%) and D168E (0.7%) in them. Amino-acid substitutions resistant to BMS-790052 (L31M and/or Y93H) were detected in 33 of the 294 (11.2%) patients, and Y93H (8.2%) predominated over L31M (2.7%). One of the 239 (0.4%) patients, who had been examined for amino-acid substitutions in both NS3 and NS5A regions, possessed HCV-1b variants resistant to NS3 inhibitors (T54S) and BMS-790052 (L31M).
Conclusions: Mutations conferring resistance to NS3 inhibitors or BMS-790052 were frequent in our treatment-naive study population, but double mutants with possible resistance to both drugs were rare. Since single mutations did not result in treatment failure in a previous pilot trial combining BMS-790052 and an NS3 inhibitor, larger trials of this drug regimen appear warranted in the Japanese population.

1. Background

Worldwide, an estimated 170 million people are infected with hepatitis C virus (HCV) persistently,[1] and approximately one-third of them develop life-threatening liver diseases, such as decompensated cirrhosis and hepatocellular carcinoma.[2] The triple therapy with an NS3 protease inhibitor, telaprevir or boceprevir, in combination with pegylated (PEG)-interferon (IFN) and ribavirin (RBV), has increased sustained virological response (SVR) to about 70% in the patients with HCV of genotype 1b (HCV-1b).[3–7] Still, approximately 30% of them fail to clear HCV by the triple therapy, and, in addition, many more cannot receive it because of contraindications, such as advanced ages, anaemia and co-morbid conditions.

Recently, a combination therapy with two direct-acting antivirals (DAAs), which are targeted to different regions in the viral genome, was introduced to treatment of patients with HCV-1b, and has gained promising results. Thus, a second-generation NS3 protease inhibitor (BMS-650032 [asunaprevir]) combined with an NS5A inhibitor (BMS-790052 [daclatasvir]) for 24 weeks induced SVR in two of the two,[8] as well as in 10 of the 10,[9] patients with HCV-1b with excellent safety profiles.

Abbreviations: HCV, hepatitis C virus; IFN, interferon; SOC, standard-of-care; PEG, pegylated; RBV, ribavirin; SVR, sustained virological response; DAA, direct-acting antiviral.

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2. Objectives

For extending the combination treatment with BMS-650032 and BMS-790052 to many more patients with HCV-1b, it is necessary to examine how frequently viral variants, which have resistance to NS3 protease inhibitors or NS5A inhibitors,[10–13] occur in patients with HCV-1b.

3. Study design

3.1. Patients

During 2000 through 2010, sera were obtained from the 362 patients with HCV-1b at the Department of Hepatology in Toranomon Hospital in Tokyo, and had been stored frozen at −80 °C. They all were treatment-naive to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052); 134 of them (37.0%) had received IFN-based treatments previously. The nucleotide sequence of the NS3 region in HCV RNA was determined in 307 patients and that of NS5A region in 294, and sequences of both NS3 and NS5A were determined in 239.

3.2. Sequencing NS3 and NS5A regions

HCV RNA was amplified by polymerase chain reaction with appropriate nested primers in NS3[14] or NS5A[15] region, and sequences of the N-terminal 609 nucleotides in the NS3 region and those of the N-terminal 600 nucleotides in the NS5A region were determined by the direct sequencing method. The major sequences were adopted, which would represent the consensus sequence. They have been deposited in the Genbank under the accession numbers AB693834–AB693872 and AB709241–AB709802.

3.3. Amino-acid substitutions for the resistance to NS3 protease inhibitors or the NS5A inhibitor (BMS-790052)

V36A/M/L/G, T54A/S, V55A, Q80K/R/H/G, R155K/T/I/M/G/L/S/Q, A156V/T/S/I/G, D168A/V/E/G/N/T/Y/H/I and V170A have been identified as amino-acid substitutions resistant to NS3 protease inhibitors, including linear ketoamids (telaprevir, boceprevir, SCH900518 and BI201335) and macrocyclic compounds (MK7009, TMC435350, ITMN191, GS-9256, ABT450 and BMS-791325).[16,17] L31M and Y93H have been recognised as the most powerful substitutions in HCV-1b for the resistance to BMS-790052.[18–21]

4. Results

4.1. Baseline characteristics of patients with HCV-1b who were naive to DAAs

Table 1 lists the baseline characteristics of the 362 patients infected with HCV-1b. Of them, 134 (37.0%) had received IFN-based treatments previously, including 78 (21.6%) with IFN monotherapy and 56 (15.4%) given combination therapy with IFN or PEG-IFN and RBV. Liver biopsies had been performed on 201 of the 362 (55.5%) patients. The majority of them (47.5%) had fibrosis stages ≤F2, by the classification of Desmet et al.,[22] and none had cirrhosis.

4.2. Amino-acid substitutions for the resistance to NS3 inhibitors or the NS5 inhibitor (BMS-790052)

Table 2 shows frequencies of amino-acid substitutions for the resistance to NS3 inhibitors in 307 patients. Of them, 15 (4.9%) were infected with HCV-1b variants having V36A, T54S, Q80R or D168E, and T54S predominated over Q80R, V36A and D168E. Resistance profiles are comparable between Japanese patients in this study and 366 European and 34 American patients (total: 400 patients) retrieved from the Genbank.

Table 3 shows frequencies of amino-acid substitutions for the resistance to the NS5 inhibitor (BMS-790052) in the 294 patients. Y93H predominated over L31M, and one patient had both Y93H and L31M. Overall, 33 (11.2%) of them were infected with HCV-1b variants with L31M or Y93H, or both. One of the 239 (0.4%) patients, for whom both NS3 and NS5A sequences had been examined, was infected with HCV-1b variants with resistance to NS3 inhibitors (T54S) and NS5A inhibitor (L31M).

Table 1
Baseline characteristic of the patients infected with HCV of genotype 1b who were naive to direct-acting antivirals.

Demographic data	(n=362)
Male (%)	213 (58.8%)
Age (years)	55 (18–75)
IFN-based treatments	
Treatment-naive	228 (63.0%)
IFN monotherapy	78 (21.6%)
IFN (or PEG-IFN) plus ribavirin	56 (15.4%)
Laboratory data	
Alanine aminotransferase (IU/L)	54 (12–348)
Asparatate aninotransferase (IU/L)	41 (17–350)
Platelets ($\times 10^3/mm^3/\mu L$)	174 (64–366)
HCV RNA (log IU/mL)	6.7 (<1.2 to >7.6)
Stage of liver fibrosis[a]	(n=201)
F1	117 (58.2%)
F2	55 (27.4%)
F3	29 (14.4%)
F4	0

Values are the number with percentage in parentheses or the mean with range in parentheses.

[a] Classified by the criteria of Desmet et al.[22]

Table 2
Substitutions of amino acids in the NS3 protease region for the resistance to NS3 inhibitors in Japanese patients in the present study and in European or American patients with HCV-1b retrieved from the Genbank.

Substitutions	This study (n=307) n(%)	Database[a] (n=400) n(%)
V36A	1 (0.3%)	1 (0.3%)
T54A	0	1 (0.3%)
T54S	10 (3.3%)	5 (1.2%)
V55A	0	1 (0.3%)
Q80R	2 (0.7%)	16 (4.0%)
A156T	0	1 (0.3%)
D168E	2 (0.7%)	2 (0.5%)
V170A	0	2 (0.5%)
Total	15 (4.9%)	29 (7.3%)

[a] HCV-1b sequences were retrieved from the Genbank. There were 400 sequences in total, exclusive of repetitive sequences, including 307 from France, 53 from Spain, 6 from Germany and 34 from USA.

Table 3
Substitutions of amino acids in the NS5A region for the resistance to BMS-790052 in Japanese patients in the present study and in patients with HCV-1b retrieved from the European HCV database.

Substitutions	This study (n=294) n(%)	Database[a] (n=1796) n(%)
L31M	8 (2.7%)	68 (3.8%)
L31V	0	38 (2.1%)
Y93H	24 (8.2%)	149 (8.3%)
Y93H/L31M	1 (0.3%)	Unknown
Total	33 (11.2%)	255 (14.2%)

[a] The sequences of HCV-1b were retrieved from the European HCV database and reported by Fridell et al.[18]

Factors influencing HCV-1b variants resistant to NS3 inhibitors or BMS-790052 were evaluated by univariate analysis with use of the Statistical Package for Social Sciences (SPSSII v.11.0, IBM Co., Chicago, IL, USA). None of age, sex, transaminase levels, platelet counts, HCV RNA loads and histological stages increased the prevalence of HCV-1b variants resistant to either of these two kinds of DAAs.

5. Discussion

DAAs have different antiviral targets and distinct resistance profiles that are dependent on HCV genotypes/subtypes.[16,21,23] For treatment of patients with HCV-1b, a combination of a second-generation NS3 protease inhibitor (BMS-650032) and an NS5A inhibitor (BMS-790052) has gained SVR in two of the two, as well as 10 of the 10, patients with HCV-1b.[8,9] By contrast, the combination therapy was less effective in the nine patients with HCV-1a, and viral breakthroughs occurred in six (67%) of them.[8] In HCV-1a, only one nucleotide mutation gives rise to amino-acid substitutions resistant to NS3 protease inhibitors (R155K/T/S/M/I), instead of two required in HCV-1b,[23] which would be responsible, at least in part, for poor responses to the combination therapy in patients with HCV-1a.

There is a possibility that HCV-1b variants resistant to both BMS-650032 and BMS-790052 may be selected during the combination therapy, and result in viral breakthroughs during treatment. Of the 307 patients, who had been examined, 15 (4.9%) were infected with HCV-1b with amino-acid substitutions for the resistance to NS3 protease inhibitors. Of the NS3 resistance mutations detected, only D168E is relevant to the second-generation protease inhibitors,[16,17] and, therefore, only 0.7% of the treatment-naive patients carried relevant resistance mutations when focussing on a possible combination of BMS-650032 with other DAAs. It needs to be pointed out that a possibility remains for the presence of minor HCV populations with resistance to DAAs that might have escaped the detection by direct sequencing.

HCV-1b variants with L31M or Y93H, which confers strong resistance to the NS5A inhibitor (BMS-790052),[20] were detected in 33 of the 294 (11.2%) patients with HCV-1b; one of them was infected with variants with both L31M and Y93H. Such a frequency is comparable to those in 1796 patients from the European HCV database (L31M, 5.9%; Y93C/H, 8.4%).[18] Variants with Y93H were detected in 3 of the 10 (30%) patients receiving the combination therapy with BMS-650032 and BMS-790052.[9] Since they all gained SVR, variants with Y93H alone, in the absence of those resistant to macrocyclic NS3 protease inhibitors, would not cause treatment failure in the patients who receive the combination therapy. Co-occurrence of variants resistant to NS3 protease inhibitors and those to the NS5A inhibitor was observed in only one of the 239 (0.4%) patients for whom both of them were examined. They may or may not exist on the same virion, because they were detected by direct sequencing. Therefore, results suggest that most patients with HCV-1b in our geographic area can be good candidates to succeed in resolving infection after combination therapy with NS3 inhibitors and BMS-790052.

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Competing interest: Dr. Kumada reports having received investigator, lecture and consulting fees from Bristol-Myers KK. No other potential conflicts of interest relevant to this article were reported.

Ethical approval: Informed consent was obtained from each patient.

References

1. http://www.who.int/mediacentre/factsheets/fs164/en/. Accessed August 2011.
2. Seeff LB. Natural history of chronic hepatitis C. *Hepatology* 2002;**36**: S35–46.
3. Hezode C, Forestier N, Dusheiko G, Ferenci P, Pol S, Goeser T, et al. Telaprevir and peginterferon with or without ribavirin for chronic HCV infection. *N Engl J Med* 2009;**360**:1839–50.
4. Kumada H, Toyota J, Okanoue T, Chayama K, Tsubouchi H, Hayashi N. Telaprevir with peginterferon and ribavirin for treatment-naive patients chronically infected with HCV of genotype 1 in Japan. *J Hepatol* 2012;**56**: 78–84.
5. McHutchison JG, Everson GT, Gordon SC, Jacobson IM, Sulkowski M, Kauffman R, et al. Telaprevir with peginterferon and ribavirin for chronic HCV genotype 1 infection. *N Engl J Med* 2009;**360**:1827–38.
6. Bacon BR, Gordon SC, Lawitz E, Marcellin P, Vierling JM, Zeuzem S, et al. Boceprevir for previously treated chronic HCV genotype 1 infection. *N Engl J Med* 2011;**364**:1207–17.
7. Poordad F, McCone Jr J, Bacon BR, Bruno S, Manns MP, Sulkowski MS, et al. Boceprevir for untreated chronic HCV genotype 1 infection. *N Engl J Med* 2011;**364**:1195–206.
8. Lok AS, Gardiner DF, Lawitz E, Martorell C, Everson GT, Ghalib R, et al. Preliminary study of two antiviral agents for hepatitis C genotype 1. *N Engl J Med* 2012;**366**:216–24.
9. Chayama K, Takahashi S, Toyota J, Karino T, Ikdeda K, Ishikawa H, et al. Dual therapy with the NS5A inhibitor BMS-790052 and the NS3 protease inhibitor BMS-650032 in HCV genotype 1b-infected null responders. *Hepatology* 2012;**55**:742–8.
10. Bartels DJ, Zhou Y, Zhang EZ, Marcial M, Byrn RA, Pfeiffer T, et al. Natural prevalence of hepatitis C virus variants with decreased sensitivity to NS3.4A protease inhibitors in treatment-naive subjects. *J Infect Dis* 2008;**198**: 800–7.
11. Cubero M, Esteban JI, Otero T, Sauleda S, Bes M, Esteban R, et al. Naturally occurring NS3-protease-inhibitor resistant mutant A156T in the liver of an untreated chronic hepatitis C patient. *Virology* 2008;**370**:237–45.
12. Gaudieri S, Rauch A, Pfafferott K, Barnes E, Cheng W, McCaughan G, et al. Hepatitis C virus drug resistance and immune-driven adaptations: relevance to new antiviral therapy. *Hepatology* 2009;**49**:1069–82.
13. Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. *Hepatology* 2008;**48**: 1769–78.
14. Suzuki F, Suzuki Y, Akuta N, Sezaki H, Yatsuji H, Arase Y, et al. Sustained virological response in a patient with chronic hepatitis C treated by monotherapy with the NS3-4A protease inhibitor telaprevir. *J Clin Virol* 2010;**47**: 76–8.
15. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 2008;**48**:38–47.
16. Halfon P, Locarnini S. Hepatitis C virus resistance to protease inhibitors. *J Hepatol* 2011;**55**:192–206.
17. Romano KP, Ali A, Royer WE, Schiffer CA. Drug resistance against HCV NS3/4A inhibitors is defined by the balance of substrate recognition versus inhibitor binding. *Proc Natl Acad Sci U S A* 2010;**107**:20986–91.
18. Fridell RA, Qiu D, Wang C, Valera L, Gao M. Resistance analysis of the hepatitis C virus NS5A inhibitor BMS-790052 in an in vitro replicon system. *Antimicrob Agents Chemother* 2010;**54**:3641–50.
19. Fridell RA, Wang C, Sun JH, O'Boyle 2nd DR, Nower P, Valera L, et al. Genotypic and phenotypic analysis of variants resistant to hepatitis C virus nonstructural protein 5A replication complex inhibitor BMS-790052 in humans: in vitro and in vivo correlations. *Hepatology* 2011;**54**:1924–35.
20. Gao M, Nettles RE, Belema M, Snyder LB, Nguyen VN, Fridell RA, et al. Chemical genetics strategy identifies an HCV NS5A inhibitor with a potent clinical effect. *Nature* 2010;**465**:96–100.
21. Scheel TK, Gottwein JM, Mikkelsen LS, Jensen TB, Bukh J. Recombinant HCV variants with NS5A from genotypes 1-7 have different sensitivities to an NS5A inhibitor but not interferon-alpha. *Gastroenterology* 2011;**140**: 1032–42.
22. Desmet VJ, Gerber M, Hoofnagle JH, Manns M, Scheuer PJ. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology* 1994;**19**: 1513–20.
23. Sarrazin C, Zeuzem S. Resistance to direct antiviral agents in patients with hepatitis C virus infection. *Gastroenterology* 2010;**138**:447–62.

Research Article

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Long-term continuous entecavir therapy in nucleos(t)ide-naïve chronic hepatitis B patients

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Background & Aims: We determined the antiviral potency and viral resistance rate after 4 years of continuous entecavir treatment in patients with chronic hepatitis B (CHB) infection.

Methods: The cumulative rates of undetectable hepatitis B virus DNA (HBV DNA; <2.6 $\log_{10}$ copies/ml), hepatitis B e antigen (HBeAg) seronegativity, seroconversion, alanine aminotransferase (ALT) normalization, and entecavir signature mutations were calculated in 474 nucleos(t)ide-naïve CHB patients (HBeAg-positive: 47%) on continuous entecavir treatment for 4 years.

Results: Median age was 47 years and follow-up period was 2.4 years, with 403, 281, 165, and 73 patients followed-up for at least 1, 2, 3, and 4 years, respectively. Incremental increases were observed in the rates of undetectable HBV DNA, HBeAg seroclearance and seroconversion, and ALT normalization, reaching 96%, 42%, 38% and 93%, respectively, by the fourth year. In all, 100% and 93% of patients negative and positive for HBeAg, respectively, had undetectable HBV DNA at year 4. Of 165 patients, HBV DNA was detectable in nine patients after 3 years. Multivariate analysis identified HBV DNA level ($\leqslant$7.6 $\log_{10}$ copies/ml, OR = 15.8; 95% CI = 43.1–79.9, P = 0.001) as an independent predictor of undetectable HBV DNA at year 3. Five patients experienced virological breakthrough including two (0.4%) who developed entecavir-resistance mutations.

Conclusions: Continuous treatment of nucleos(t)ide-naïve CHB patients with entecavir over 4 years was associated with 96% chance of undetectable HBV DNA and only 0.4% chance of emerging entecavir-resistant mutations.

Keywords: Hepatitis B virus; Entecavir; Resistance; Virological breakthrough.
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Abbreviations: AFP, α fetoprotein; ALT, alanine aminotransferase; AST, aspartate transaminase; CHB, chronic hepatitis B; CIs, confidence intervals; HBeAg, hepatitis B e antigen; HBsAg, hepatitis B virus surface antigen; HBV, hepatitis B virus; HBV DNA, hepatitis B virus DNA; ORs, odds ratios; PCR, polymerase chain reaction; ULN, upper limit of normal; VBT, virologic breakthrough.

Introduction

Approximately 350–400 million people worldwide have chronic hepatitis B (CHB) infection, the majority of whom live in the Asia–Pacific region [1,2]. CHB patients with elevated viral load are at risk of developing cirrhosis, liver failure, and hepatocellular carcinoma.

Recent investigations have shown that entecavir suppressed HBV DNA replication to undetectable levels and normalized alanine aminotransferase (ALT) levels in nucleos(t)ide-naive CHB patients in Japan and other countries [3–10]. In addition, genotypic resistance to long-term entecavir monotherapy remained rare [5,6,9,10]. To date, there are two 5-year studies [6,8] and two 3-year studies [7,9] of entecavir therapy for nucleos(t)ide-naïve patients. Both studies stemmed from extension studies with the original cohorts from two large-scale phase III trials of treatment-naïve patients [3,4]. In these trials, patients were administered 0.5 mg entecavir for 1 year and later divided into three categories: (i) complete responders, defined as patients with HBV DNA $<7 \times 10^5$ copies/ml and ALT level <1.25 times the upper limit of normal (ULN) for hepatitis B e antigen (HBeAg)-negative patients and an additional loss of HBeAg for HBeAg-positive patients; (ii) non-responders, defined as HBV DNA $\geqslant 7 \times 10^5$ copies/ml; and, (iii) virological responders, defined as HBV DNA $<7 \times 10^5$copies/ml and ALT >1.25× ULN regardless of HBeAg status or persistent HBeAg for HBeAg-positive patients. Treatment was terminated in the complete responders but continued in virological responders. Non-responders were provided additional therapy in a rollover study in which some patients were initially treated with a combination of 1 mg entecavir and lamivudine for several months before receiving 1 mg entecavir as monotherapy. Furthermore, a substantial proportion of complete responders relapsed after various intervals following cessation of therapy and they were also assigned to a rollover study receiving 1 mg entecavir monotherapy. Because of these strict protocols, the precise viral-suppression and drug-resistance data for treatment-naïve patients who were treated continuously with 0.5 mg entecavir daily (the recommended dosage) remain unavailable.

The aims of this cohort study were (1) to investigate the efficacy of entecavir in clinical practice beyond 4 years for nucleos(t)ide-naïve CHB and cirrhosis patients, (2) to explore baseline factors associated with virological response to entecavir,

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and (3) to investigate virological breakthrough during long-term entecavir treatment.

Patients and methods

Study population

We performed a retrospective analysis of 474 CHB and cirrhosis patients who received entecavir treatment at the Department of Hepatology, Toranomon Hospital, Tokyo, from March 2004 to May 2011, and adhered to the treatment for more than 6 months (Table 1). All patients were negative for hepatitis C serological markers, but all had detectable HBV surface antigen (HBsAg) for at least 6 months prior to the start of entecavir therapy. Two patients received 0.01 mg entecavir and one patient received 0.1 mg entecavir for 24 weeks, prior to 0.5 mg/day from a phase II study ETV-047 in Japan [11]. The other patients received 0.5 mg entecavir. None had received other nucleos(t)ide analogs. The diagnosis of chronic hepatitis and cirrhosis was established by needle biopsy, peritoneoscopy, or clinically before treatment. The clinical criteria for chronic hepatitis included elevated ALT levels over 6 months and absence of clinical evidence of portal hypertension, such as esophageal varices, ascites, hepatic encephalopathy, and features suggestive of cirrhosis on ultrasonography. Chronic hepatitis and cirrhosis were diagnosed in 374 and 102 patients, respectively. Twenty-eight patients were lost to follow-up, including 10 patients who moved to other locations, seven who never visited the hospital again, two who became pregnant, four who died, four who had virological breakthrough (VBT), and one who showed disappearance of HBsAg. Moreover, 18 patients developed HCC during treatment and their data until loss to follow-up or diagnosis of HCC were analyzed. Informed consent was obtained from each patient enrolled in the study and the study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the Toranomon Hospital Ethical Committee.

Analysis of treatment efficacy

The clinical efficacy of entecavir was assessed as the proportion of patients who achieved HBV DNA suppression to undetectable levels (<2.6$\log_{10}$ copies/ml), and those who achieved ALT normalization (<1 × ULN). HBV DNA was measured using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, lower limit of detection of 2.6$\log_{10}$ copies/ml) [12]. HBeAg seroclearance and seroconversion were also analyzed. Measurements were made on stored samples taken at baseline and every year after that since entecavir treatment initiation.

Statistical analysis

Differences between groups were examined for statistical significance using the χ^2 test where appropriate. Spearman correlation coefficient (two-tailed) was used to evaluate the correlation between albumin and other factors. Independent predictive factors associated with response to entecavir treatment were determined using multivariate multiple logistic regression. The following 12 potential predictors of response to entecavir treatment were assessed in this study: age, sex, severity of liver disease (CH or cirrhosis), HBV genotype, as well as levels of aspartate transaminase (AST), ALT, bilirubin, albumin, platelets, α fetoprotein (AFP), HBeAg, and HBV DNA. All factors found to be at least marginally associated with undetectable levels of HBV DNA after 1–4 years (p <0.10) were entered into the multivariate multiple logistic regression analysis. The above calculations were performed using The Statistical Package for Social Sciences version 11.0.1J (SPSS Inc., Chicago, IL).

The odds ratios (ORs) and 95% confidence intervals (CIs) were calculated to assess the relative risk confidence. Independent risk factors predicting achievement of HBeAg seroclearance and seroconversion were analyzed using stepwise Cox regression analysis. Potential factors that could predict achievement of HBeAg seroclearance assessed here were the above 11 variables, each transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. All factors found to be at least marginally associated with HBeAg seroclearance and seroconversion (p <0.10) were tested in the multivariate Cox proportional hazard model. A Kaplan–Meier estimate was performed using the SPSS software, and p values were calculated using the Cox-Mantel log-rank test. The Mann–Whitney U test was used for comparison of HBV DNA levels in patients with seroconversion to those with seroclearance. A two-tailed p value <0.05 was considered statistically significant.

Table 1. Characteristics of patients at the start of entecavir therapy. Table data are number of patients or median (range).

Demography	
n	474
Sex, male/female	321/153
Age, yr	47 (17-82)
Family history of HBV	291 (61%)
Cirrhosis	102 (22%)
Median duration of treatment, yr (range)	2.37 (0.5-7.2)
Laboratory data	
AST, IU/L	52 (14-1595)
ALT, IU/L	70 (8-2121)
Bilirubin, mg/dl	0.7 (0.2-3.9)
γ-GTP, IU/L	38 (9-679)
Albumin, g/dl	3.9 (1.9-5.1)
Alpha fetoprotein, ng/ml	5 (1-379)
Viral load, $\log_{10}$ copies/ml	6.7 (<2.6->9.0)
HBeAg-positive	222 (47%)
HBV genotypes, A/B/C/H/unknown	12/67/336/2/57

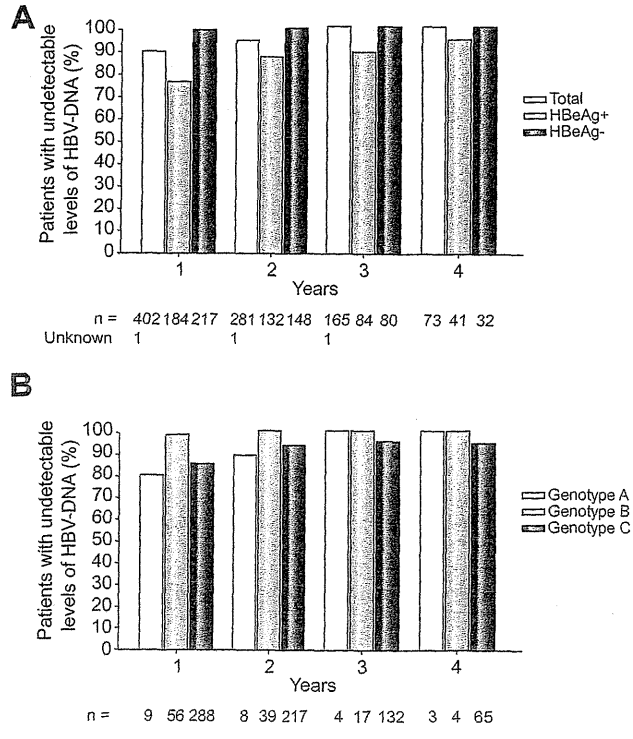

Fig. 1. Percentages of patients who had undetectable levels of HBV DNA between years 1 through 4. (A) HBeAg-positive and negative patients and (B) patients with genotype A, B, or C.

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Table 2. Univariate and multivariate analyses of host and viral factors associated with undetectable levels of HBV DNA at year 1.

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Sex (female)	1.06 (0.56-2.02)	0.842		
Age (>40 yr)	1.85 (1.0-3.4)	0.047		
Cirrhosis (present)	2.39 (0.98-5.81)	0.048		
Albumin (>4 g/dl)	1.28 (0.63-2.62)	0.494		
Bilirubin (>1.2 g/dl)	1.63 (0.56-4.76)	0.366		
ALT (>5 x IU/L)	4.57 (1.38-15.1)	0.007	11.9 (3.3-41.7)	<0.001
AST (>5 x IU/L)	2.25 (0.67-7.53)	0.178		
γ-GTP (≤20 IU/L)	1.75 (0.60-5.08)	0.300		
AFP (>10 ng/ml)	1.63 (0.61-4.37)	0.328		
Platelets (≤10/mm^3)	2.39 (0.56-10.3)	0.288		
Genotype (B)	9.57 (1.29-70.92)	0.007		
HBeAg (negative)	23.78 (7.25-77.95)	<0.001	8.5 (2.3-31.2)	0.001
HBV DNA (≤7.6 $\log_{10}$ copies/ml)	16.5 (8.0-34.2)	<0.001	10.0 (4.3-23.1)	<0.001

Table 3. Univariate and multivariate analyses of host and viral factors associated with undetectable levels of HBV DNA at year 2.

Parameter	Univariate analysis		Multivariate analysis	
	OR (95% CI)	*p* value	OR (95% CI)	*p* value
Sex (male)	0.524 (0.169-1.627)	0.257		
Age (>40 yr)	2.825 (1.1-7.25)	0.025		
Cirrhosis (present)	3.06 (0.69-13.5)	0.173		
Albumin (≤3.5 g/dl)	4.64 (0.603-35.73)	0.134		
Bilirubin (≤0.5 g/dl)	2.80 (0.79-9.93)	0.126		
ALT (>5 x IU/L)	5.35 (0.7-40.9)	0.054	16.7 (2.0-136.8)	0.009
AST (>5 x IU/L)	2.62 (0.34-20.3)	0.298		
γ-GTP (≤100 IU/L)	1.79 (0.557-5.73)	0.304		
AFP (>15 ng/ml)	2.12 (0.27-16.95)	0.699		
Platelets (≤12/mm^3)	4.74 (0.619-36.31)	0.136		
Genotype (B)	1.082 (1.042-1.123)	0.076		
HBeAg (negative)	23.21 (3.05-176.46)	<0.001		
HBV DNA (≤7.6 $\log_{10}$ copies/ml)	39.91 (8.912-178.76)	<0.001	121.7 (15.3-965.9)	<0.001

Results

Study population

Of the 474 subjects in this study, 68% were males, and the mean age was 47 years. The mean HBV DNA level was 6.7 $\log_{10}$ copies/ml, mean ALT level was 70 IU/L, and 47% of patients were HBeAg-positive. At baseline, there were 12, 67, and 336 patients of genotype A, B, and C, respectively, and among the patients belonging to these genotypes, 4, 11, and 188, respectively, were HBeAg-positive.

Virological response

Undetectable levels of HBV DNA were identified at years 1 through 4 in 88% (353/402), 93% (262/281), 95% (156/165), and 96% (70/73) of patients, respectively (Fig. 1A). Among the HBeAg-positive patients at baseline, 75% (138/184), 86% (114/132), 89% (75/84), and 93% (38/41), and among the HBeAg-negative patients at baseline, 99% (214/217), 99% (147/148), 100% (80/80), and 100% (32/32) had undetectable levels of HBV DNA at years 1 through 4, respectively.

Among the patients with genotype A, 78% (7/9), 88% (7/8), 100% (4/4), and 100% (3/3) of patients had undetectable levels of HBV DNA at years 1 through 4, respectively (Fig. 1B). Among the HBeAg-positive patients with genotype A at baseline, 50% (2/4), 67% (2/3), 100% (2/2), and 100% (2/2) had undetectable levels of HBV DNA at years 1 through 4, respectively. Among patients with genotype B, 98% (55/56), 100% (39/39), 100% (17/17), and 100% (4/4) had undetectable levels at years 1 through 4, respectively (Fig. 1B). Among the HBeAg-positive patients with genotype B at baseline, 88% (7/8), 100% (5/5), and 100% (3/3) had

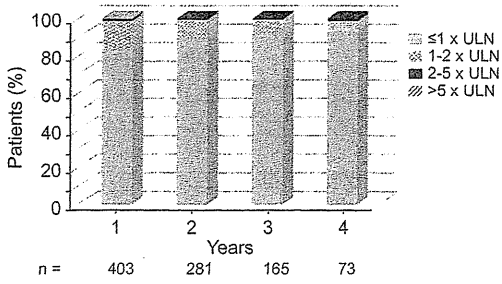

Fig. 2. Percentages of patients with ALT level <1× upper limit of normal level (ULN), <1–2× ULN, 2–5× ULN, and 5× ULN.

undetectable levels of HBV DNA at years 1 through 3, respectively. None of the patients with genotype B at baseline were HBeAg-positive at year 4. Among the patients with genotype C, 85% (246/288), 93% (201/217), 95% (125/132), and 95% (62/65) had undetectable levels of HBV DNA at years 1 through 4, respectively (Fig. 1B), and of these, 74% (116/156), 87% (102/117), 91% (67/74), and 92% (35/38), respectively, were HBeAg-positive.

Factors associated with detectable levels of HBV DNA at years 1, 2, and 3

Of the 402 patients, 353 had no detectable HBV DNA after 1 year. At the start of treatment, factors associated with undetectable levels of HBV DNA in the first year were age (>40 years, $p = 0.047$), cirrhosis (present, $p = 0.048$), ALT (>5× ULN, $p = 0.007$), genotype (B, $p = 0.007$), HBeAg (negative, $p < 0.001$), and HBV DNA level (<7.6 $\log_{10}$copies/ml, $p < 0.001$), by univariate analysis (Table 2). Multivariate analysis identified three parameters, namely ALT (>5× ULN, OR = 11.9; 95% CI = 3.3–41.7, $p < 0.001$), HBeAg (negative, OR = 8.5; 95% CI = 2.3–31.2, $p = 0.001$), and HBV DNA level (<7.6 $\log_{10}$ copies/ml, OR = 10.0; 95% CI = 4.3–23.1, $p < 0.001$).

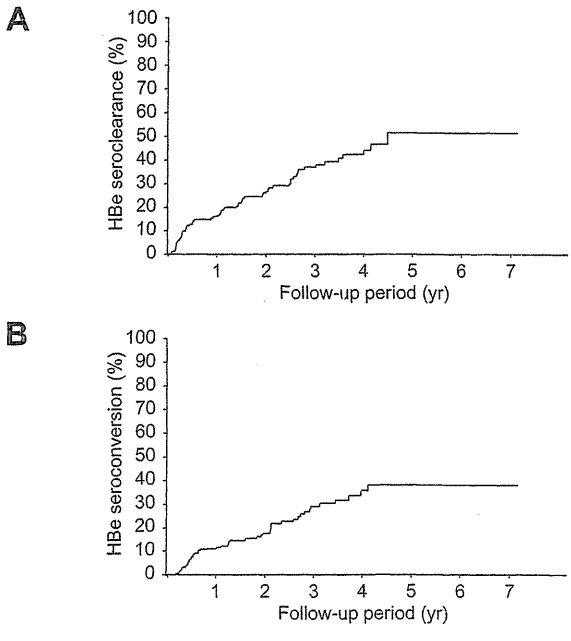

Fig. 3. Change of the HBeAg status during follow-up. Cumulative rates of (A) HBe seroclearance and (B) HBe seroconversion in HBeAg-positive patients, analyzed with the Kaplan–Meier test.

Of 281 patients, HBV DNA was undetectable in 262 patients in the second year, with univariate analysis identifying the following associated factors: age (>40 years, $p = 0.025$), ALT (>5× ULN, $p = 0.054$), HBeAg (negative, $p < 0.001$), and HBV DNA level (≤7.6 log copies/ml, $p < 0.001$). Of these, multivariate analysis identified ALT (>5× ULN, OR = 16.7; 95% CI = 2.0–136.8, $p = 0.009$) and HBV DNA level (≤7.6 $\log_{10}$ copies/ml, OR = 121.7; 95% CI = 15.3–965.9, $p < 0.001$) as significant factors (Table 3).

Of 165 patients, HBV DNA was undetectable in 156 patients in the third year, with univariate analysis identifying the following associated factors at the start of treatment: Gender (male, $p = 0.04$), HBeAg (negative, $p = 0.002$) and HBV DNA level (≤7.6 log copies/ml, $p < 0.001$). Multivariate analysis identified only HBV DNA level as significant (≤7.6 $\log_{10}$ copies/ml, OR = 15.8; 95% CI = 43.1–79.9, $p = 0.001$).

Biochemical response

The percentages of patients with normal ALT levels (<1x ULN) at years 1, 2, 3, 4 were 83% (336/403), 89% (251/281), 92% (151/165), and 93% (68/73), respectively (Fig. 2). In HBeAg-positive patients at baseline, those who achieved normal ALT levels at years 1, 2, 3, 4 were 81% (148/183), 88% (116/132), 90% (76/84), and 95% (39/41), respectively. The respective data for HBeAg-negative patients at baseline were 85% (187/219), 91% (134/148), 93% (74/80), and 91% (29/32).

HBeAg seroclearance and seroconversion

HBeAg positivity at baseline was detected in 222 patients (47%) (Table 1), and Fig. 3A shows the cumulative clearance of HBeAg calculated with the Kaplan–Meier method. The percentages of patients with seroclearance were 16%, 24%, 37%, and 42% at years 1 through 4, respectively. Univariate analysis identified the following HBeAg seroclearance-associated factors at the start of treatment: age (>40 years, $p = 0.052$), platelet count (<12 × 10^4/mm^3, $p = 0.028$), and HBV DNA (<7.0 log copies/ml, $p = 0.006$). Multivariate analysis identified HBV DNA (<7 $\log_{10}$ copies/ml, RR = 1.9; 95% CI = 1.2–3.1, $p = 0.007$) as the only significant determinant of seroclearance. Of 70 patients who achieved anti-HBe seroclearance, 52 patients achieved anti-HBe seroconversion. Fig. 3B shows the cumulative seroconversion rate of HBeAg calculated by the Kaplan–Meier test. The proportions of patients who showed seroconversion were 12%, 18%, 29%, and 38% at years 1 through 4, respectively. Univariate analysis demonstrated that age (>40 years, $p = 0.020$), albumin (<3.5 g/dl, $p = 0.021$) and platelet count (<20 × 10^4/mm^3, $p = 0.067$) correlated with HBeAg seroconversion at the start of treatment. Multivariate analysis that included the above factors identified serum albumin as the only significant determinant of seroconversion (<3.5 g/dl, RR = 2.0; 95% CI = 1.1–3.6, $p = 0.019$). One patient achieved anti-HBe seroconversion at 25 months but became positive again at 28 months. Other patients who achieved anti-HBe seroconversion did not show HBeAg reversion. One patient achieved anti-HBe seroconversion but remained HBV DNA positive (Table 4, Patient 5). Another patient remained positive for HBV

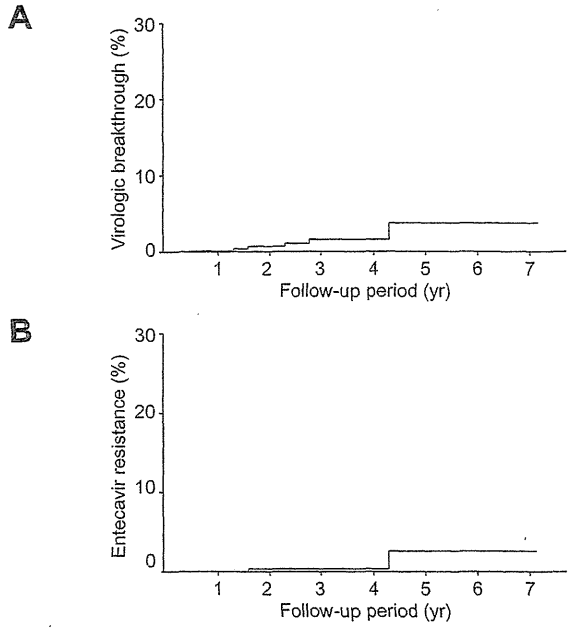

Fig. 4. Cumulative rates of patients who showed resistance to therapy analyzed with the Kaplan–Meier test. (A) Virologic breakthrough (VBT) and (B) entecavir-resistance.

DNA after anti-HBe seroconversion. One patient became negative for HBsAg at week 28.

Monitoring resistance to treatment

Five patients showed VBT during the treatment period, including two patients (Patient 1 had been reported previously [13]) who developed entecavir-resistant mutations. None of the five patients had mutation(s) for entecavir at baseline. VBT was defined as any increase in serum HBV DNA by >1 $\log_{10}$ copies/ml from nadir or redetection of serum HBV DNA at levels 10-fold the lower limit of detection of the HBV DNA assay after having an undetectable result. Table 4 shows the patient baseline demographics, HBV DNA levels, and viral resistance profiles. All patients were positive for HBeAg and had serum levels of HBV DNA >6 $\log_{10}$ copies/ml at baseline. The median period until the appearance of the mutation was 120 (68–224) weeks. Two of the 49 (4%) patients who had detectable HBV DNA at the end of the first year subsequently developed resistance to entecavir. Furthermore, 3 of 49 (6%) patients who had detectable HBV DNA at the end of the first year developed VBT. Fig. 4A and B show the cumulative percentages of VBT and entecavir-resistance cases analyzed by the Kaplan–Meier test.

Discussion

Long-term data are rare for nucleoside-naïve patients treated continuously for more than 4 years with entecavir at the recommended dose of 0.5 mg daily. The only available data [6,8] were generated from follow-up studies of two phase III registration trials [3,4] in which patients showing complete response and non-responders were taken off entecavir. In the rollover studies, entecavir was administered to these patients at 1-mg dose at varying periods after cessation of the initial treatment. This double dose of entecavir was also given to patients showing a partial virological response after 48–96 weeks of entecavir at 0.5 mg daily. The present study has several unique features addressing specific and unanswered questions about entecavir treatment. It provided long-term results with respect to antiviral potency, viral resistance, and clinical safety for treatment-naïve patients who were treated continuously with entecavir at 0.5 mg daily for 4 years. Specifically, we found excellent viral suppression with 96% of patients achieving undetectable HBV DNA levels, only 1.1% (5/475) chance of viral breakthrough, and no clinically serious side effects after 4 years of treatment.

Genotype B was a significant factor associated with undetectable HBV DNA after the first year, although there were no significant differences after subsequent years. Previous studies showed conflicting results on the effect of HBV genotype on the response to lamivudine, with genotypes A, B, and C not affecting the antiviral response to lamivudine [14–16]. However, we have previously found that 47%, 84%, and 76% of patients had undetectable HBV DNA after the third year among patients of genotype

Table 4. Characteristics of patients with virologic breakthrough.

Patient No.	1	2	3	4	5
Age (yr)/gender	40/M	28/M	39/M	51/F	64/M
At start of entecavir therapy					
HBeAg status	+	+	+	+	+
HBV DNA ($\log_{10}$ copies/ml)	>7.6	>7.6	7.2	7.2	6.2
HBV genotype	H	A	C	C	C
Viral load at maximum suppression ($\log_{10}$ copies/ml)	<2.6	<2.6	<2.6	3.1	<2.6
Time of detection of mutation (wk)	83	224	120	68	145
HBV DNA ($\log_{10}$ copies/ml), maximum	6.8	7.2	7.1	7.6	7.8
Mutational pattern	L180M+/S202G+/M204V	L180M+/T184I+/S202G+/M204V	L180M+/M204V, L180M+/M204I	A181T	A181S+/T184A+/M204I