2006.
9. Jensen DM, Morgan TR, Marcellin P, et al. Early identification of HCV genotype 1 patients responding to 24 weeks peginterferon alpha-2a (40 kd)/ribavirin therapy. Hepatology 43: 954-960, 2006.
10. Bruno S, Cammà C, Di Marco V, et al. Peginterferon alfa-2b plus ribavirin for naïve patients with genotype 1 chronic hepatitis C: a randomized controlled trial. J Hepatol 41: 474-481, 2004.
11. Mamori S, Suzuki F, Hosaka T, et al. Interferon monotherapy for patients with chronic hepatitis C and normal serum aminotransferase levels at commencement of treatment. J Gastroenterol 39: 776-782, 2004.
12. Dalgard O, Bjøro K, Hellum KB, et al. Treatment with pegylated interferon and ribavarin in HCV infection with genotype 2 or 3 for 14 weeks: a pilot study. Hepatology 40: 1260-1265, 2004.
13. Mangia A, Santoro R, Minerva N, et al. Peginterferon alfa-2b and ribavirin for 12 vs. 24 weeks in HCV genotype 2 or 3. N Engl J Med 352: 2609-2617, 2005.
14. von Wagner M, Huber M, Berg T, et al. Peginterferon-alpha-2a (40 KD) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. Gastroenterology 129: 522-527, 2005.
15. Festi D, Sandri L, Mazzella G, et al. Safety of interferon beta treatment for chronic HCV hepatitis. World J Gastroenterol 10: 12-16, 2004.
16. Iwasaki Y, Ikeda H, Araki Y, et al. Limitation of combination therapy of interferon and ribavirin for older patients with chronic hepatitis C. Hepatology 43: 54-63, 2006.
17. Arase Y, Suzuki F, Suzuki Y, et al. Side effects of combination therapy of peginterferon and ribavirin for chronic hepatitis-C. Intern Med 46: 1827-1832, 2007.
18. Kurosaki M, Enomoto N, Murakami T, et al. Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. Hepatology 25: 750-753, 1997.
19. Enomoto M, Tamori A, Kawada N, et al. Interferon-beta plus ribavirin for patients with hepatitis C virus genotype 1: a randomized pilot trial. Gut 55: 139-140, 2006.
20. Doglio A, Laffont C, Caroli-Bosc FX, et al. Second generation of the automated Cobas Amplicor HCV assay improves sensitivity of hepatitis C virus RNA detection and yields results that are more clinically relevant. J Clin Microbiol 137: 1567-1569, 1999.
21. Albadalejo J, Alonso R, Antinozzi R, et al. Multicenter evaluation of the COBAS AMPLICOR HCV assay, an integrated PCR system for rapid detection of hepatitis C virus RNA in the diagnostic laboratory. J Clin Microbiol 36: 862-865, 1998.
22. Dusheiko G, Schmilovitz-Weiss H, Brown D, et al. Hepatitis C virus genotypes: an investigation of type-specific differences in geographic origin and disease. Hepatology 19: 13-18, 1994.
23. Katamura Y, Suzuki F, Akuta N, et al. Natural human interferon beta plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus and a high viral load. Intern Med 47: 1827-1834, 2008.
24. Arase Y, Suzuki F, Sezaki H, et al. Suitable treatment period in patients with virological response during combination therapy of peginterferon and ribavirin for chronic hepatitis C. Intern Med 47: 1301-1307, 2008.

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Efficacy of switching to entecavir monotherapy in Japanese lamivudine-pretreated patients

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entecavir, hepatitis B virus, lamivudine, viral resistance.

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Abstract

Background and Aims: To assess the efficacy of switching Japanese chronic hepatitis B patients from lamivudine monotherapy to entecavir 0.5 mg/day.

Methods: A retrospective analysis was conducted on 134 patients switched to entecavir between September 2006 and February 2008 for 6 months or more. Patients were divided into three groups based on viral load at entecavir switching point (baseline < 2.6, 2.6–5.0 and > 5.0 $\log_{10}$ copies/mL).

Results: At baseline, detection of lamivudine-resistant virus was highest in patients with higher hepatitis B virus (HBV) DNA (76% *vs* 23% in ≥ 2.6 and < 2.6 $\log_{10}$ copies/mL, respectively), and in patients with longest previous exposure to lamivudine (52%, 28% and 24% for > 3 years, 1–3 years and < 1 year, respectively). Two years after entecavir switching, HBV DNA suppression to less than 2.6 $\log_{10}$ copies/mL was achieved in 100% (32/32), 92% (12/13) and 44% (4/9) of patients in the less than 2.6, 2.6–5.0 and more than 5.0 $\log_{10}$ copies/mL baseline groups, respectively. Alanine aminotransferase (ALT) normalization occurred in 76–96% and 90–100% of patients following 1 and 2 years of entecavir treatment, respectively. One patient (2.6–5.0 $\log_{10}$ copies/mL) with lamivudine-resistant mutants at baseline developed entecavir resistance at week 48 during follow up.

Conclusion: Switching to entecavir 0.5 mg/day achieves or maintains undetectable HBV DNA levels and ALT normalization over 2 years, especially in patients with viral load less than 5.0 $\log_{10}$ copies/mL.

Introduction

Hepatitis B virus (HBV) infection is a serious public health threat affecting 350–400 million people worldwide, the majority of whom live in the Asia–Pacific region.[1,2] Chronically-infected people are at risk of developing cirrhosis, liver failure and hepatocellular carcinoma. Studies have suggested that high serum HBV DNA is a key risk predictor of chronic hepatitis B (CHB) complications.[3,4] Therefore, the main purpose of CHB therapies is to permanently suppress viral replication and sustain viral suppression to prevent long-term liver damage.[2,5,6]

Lamivudine was the first nucleoside analog to be widely prescribed for CHB patients, mainly due to its antiviral efficacy and safety profile.[2] However, lamivudine's long-term efficacy is diminished by the emergence of drug-resistant substitutions, generally in the tyrosine–methionine–aspartate–aspartate (YMDD) motif of the reverse transcriptase (rt) polymerase gene.[7–9] Detection of lamivudine-resistant HBV substitutions occurs in 15–30% and 70% of patients after 1 and 5 years of treatment, respectively.[8] Continuing lamivudine monotherapy in the presence of lamivudine resistance is not recommended because it is no longer effective in suppressing viral replication.[2] Furthermore, the initial improvement in histology and clinical benefits may be reversed or decreased due to the emergence of lamivudine-resistant substitutions.

Antiviral efficacy of entecavir (0.5 mg/day) as first-line therapy was superior to lamivudine in treatment-naïve patients on all virological, biochemical and histological end-points after 48 weeks of treatment,[10–14] with very low rates of emergence of viral resistance (1.2% after 5 years of entecavir treatment).[15,16] Entecavir has a high genetic barrier to resistance,[17–19] requiring multiple substitutions (including YMDD mutations) to express viral resistance.[16–21] In agreement with this, entecavir-resistant mutants emerge more frequently in lamivudine-refractory patients.[22,23] In a study of hepatitis B e antigen (HBeAg)-positive lamivudine-refractory patients with high HBV DNA levels at baseline (mean > 9 $\log_{10}$ copies/mL), switching to entecavir 1 mg/day achieved HBV DNA suppression to undetectable levels (< 300 copies/mL; 40%, 96 weeks) and alanine aminotransferase (ALT) normalization (81%, 96 weeks) at higher proportions than continued lamivudine

monotherapy,[22] although response to therapy was less pronounced than in treatment-naïve patients with comparable baseline levels of HBV DNA.[10,13,14] The probability of achieving HBV DNA suppression to undetectable levels at 96 weeks with entecavir was 73% in patients whose baseline HBV DNA was less than 7 $\log_{10}$ copies/mL ($n = 11$), and none of these patients developed entecavir resistance.[22]

In a randomized controlled trial of lamivudine-refractory Japanese patients with mean HBV DNA at baseline of 7.6–7.7 $\log_{10}$ copies/mL, switching to entecavir (0.5 or 1 mg/day) for 48 weeks achieved HBV DNA suppression to below detectable levels in 33% of patients in the entecavir dose groups, and ALT normalization in 78–86%.[24] Switching to entecavir in patients with evidence of lamivudine-resistant substitutions and low viral load at switching point has not been prospectively investigated in Japanese patients. There are limited data concerning the efficacy of entecavir in lamivudine-pretreated patients who have not developed lamivudine resistance.

The objective of this study was to assess the efficacy of switching to entecavir 0.5 mg/day in Japanese lamivudine-pretreated patients whose HBV DNA levels at switching point (baseline) ranged from less than 2.6 to 7.6 $\log_{10}$ copies/mL, with or without lamivudine-resistant substitutions.

Methods

Design and setting

A retrospective analysis of a CHB patient population ($n = 134$) at Toranomon Hospital (Tokyo, Japan) was performed to identify patients switched from lamivudine 100 mg/day monotherapy to entecavir 0.5 mg/day between September 2006 and February 2008, and who had received entecavir for at least 6 months. Among all patients selected, only one had a history of adefovir add-on therapy prior to switching to entecavir (case report). Conserved serum from all patients was analyzed to determine baseline characteristics and study end-points.

Study end-points

Clinical efficacy of entecavir was assessed as the proportion of patients achieving HBV DNA suppression to undetectable levels (< 400 copies/mL or < 2.6 $\log_{10}$ copies/mL), and patients achieving ALT normalization (normal ALT levels: men 8–42 IU/L, women 6–27 IU/L). HBV DNA was measured using the polymerase chain reaction (PCR)-based Amplicor HBV Monitor assay (Roche Diagnostics, Indianapolis, IN, USA; lower limit of detection of < 2.6 $\log_{10}$ copies/mL).[25] HBeAg loss in patients who were HBeAg-positive at baseline was also analyzed. Measurements were made from conserved samples taken at baseline, and after 6 months, 1 and 2 years from entecavir treatment initiation.

Assessment of viral resistance

Conserved serum was used to detect the presence of viral lamivudine-resistant rtM204V/I substitutions in all patients at baseline, and following the entecavir switch in patients treated with entecavir for at least 6 months. Lamivudine-resistant virus (rtM204V/I or YMDD motif substitutions) was analyzed using a combination of the quantitative enzyme-linked immunosorbent assay standardized using a purified *Taenia solium* cysticerci fraction (PCR enzyme-linked immunosorbent assay) and the enriched PCR enzyme linked minisequence assay.[26] Direct sequencing of HBV DNA polymerase reverse transcriptase site was also performed.[27] Detection of entecavir-resistant virus was conducted using direct sequencing of HBV DNA polymerase reverse transcriptase site.[27]

Data analyses

Statistical comparisons between treatment groups were assessed using χ^2-test and Kruskal–Wallis test where appropriate. Calculations were performed using StatView software (ver. 4.5J; Abacus Concepts, Berkeley, CA, USA). A two-tailed P-value less than 0.05 was considered statistically significant.

To identify predictive factors of HBV DNA negativity (suppression to below detectable levels) after 6 months of the entecavir switch, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors at baseline included: sex; age; levels of aspartate aminotransferase (AST), ALT, albumin, γ-glutamyl transpeptidase, total bilirubin and α-fetoprotein; platelet count; viral load; liver disease stage (cirrhosis or other); family history; HBV genotype; lamivudine treatment duration prior to entecavir switch; HBeAg status; and lamivudine resistance. Each variable was transformed into categorical data consisting of two simple ordinal numbers. All factors that were at least marginally associated with HBV DNA negativity ($P < 0.10$) were used in a multiple logistic regression analysis. To assess relative risk confidence, odds ratio (OR) and 95% confidence interval (CI) were calculated. All analyses were performed using SPSS II software ver. 11.0 (SPSS, Chicago, IL, USA).

Results

Patient characteristics before switching to entecavir

Lamivudine-pretreated patients switched to entecavir 0.5 mg/day ($n = 134$) were divided into three groups based on their HBV DNA level at the switching point: HBV DNA of less than 2.6 $\log_{10}$ copies/mL ($n = 92$), 2.6–5.0 $\log_{10}$ copies/mL ($n = 25$) and more than 5.0 $\log_{10}$ copies/mL ($n = 17$) (Table 1). Patients with HBV DNA levels of more than 5.0 $\log_{10}$ copies/mL had the highest AST/ALT levels and highest proportion of HBeAg-positive cases ($P < 0.05$). These patients had been treated with lamivudine for the shortest time period compared to patients from the two other groups ($P < 0.05$; Table 1).

Viral resistance to lamivudine at baseline

At baseline, lamivudine-resistant rtM204V/I mutant virus was detected in 23% of patients with HBV DNA of less than 2.6 $\log_{10}$ copies/mL, compared to 76% in each of the HBV DNA 2.6–5.0 $\log_{10}$ copies/mL and more than 5.0 $\log_{10}$ copies/mL groups (Table 2). In all treatment groups, a higher occurrence of resistant virus was observed with longer exposure to lamivudine, independent of viral DNA levels.

Table 1 Patient characteristics at point of switching to entecavir (baseline) and entecavir treatment duration

	All patients	Serum HBV DNA levels by baseline treatment group, log_{10} copies/mL			
		< 2.6	2.6–5.0	> 5.0	*P**
Patients, *n*	134	92	25	17	
Sex, *n* male/female	94/40	67/25	19/6	8/9	0.08
Age, years†	53 (23–83)	53 (27–83)	50 (32–77)	37 (23–77)	0.036
Bilirubin, mg/dL†	0.6 (0.2–3.4)	0.6 (0.2–3.4)	0.6 (0.3–1.8)	0.7 (0.3–1.2)	0.53
AST, IU/L†	24 (13–451)	23 (13–53)	23 (14–50)	37 (14–451)	0.0083
ALT, IU/L†	21 (8–1382)	21 (8–56)	20 (10–111)	46 (9–1382)	0.0002
Albumin, g/dL†	3.9 (2.7–4.8)	3.9 (2.7–4.4)	4.0 (3.3–4.8)	3.9 (3.6–4.6)	0.94
Histology, *n* CH/LC	89/45	56/36	19/6	14/3	0.11
HBeAg, *n* ±	30/104	11/81	5/20	14/3	< 0.0001
HBV DNA, log_{10} copies/mL†	< 2.6 (< 2.6–7.6)	< 2.6	3.9 (2.7–5.0)	6.5 (5.1–7.6)	–
Genotype, *n* A/B/C/unknown	3/9/115/7	2/6/78/6	1/2/22/0	0/1/15/1	0.87
Treatment duration, months†					
Lamivudine	36 (0.5–103)	36 (3–103)	70 (2–89)	17 (0.5–89)	0.009
Entecavir†	21 (6–33)	20 (6–33)	24 (6–32)	27 (6–33)	0.034

*Comparison of the three patient subgroups using the Kruskal–Wallis test; $P < 0.05$ was considered statistically significant.
†Data are median (range).
‡Entecavir treatment duration is from point of switching.
ALT, alanine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B early antigen; HBV, hepatitis B virus; LC, liver cirrhosis.

Table 2 rtM204V/I mutant occurrence at baseline of switching to entecavir

	Duration of previous lamivudine treatment, years			All patients
	< 1	1–3	≥ 3	
Baseline treatment group				
< 2.6 log_{10} copies/mL	1/10 (10%)	4/35 (11%)	16/47 (34%)	23%
2.6–5.0 log_{10} copies/mL	1/5 (20%)	3/4 (75%)	15/16 (94%)	76%
> 5.0 log_{10} copies/mL	3/6 (50%)	6/7 (86%)	4/4 (100%)	76%
All patients	24%	28%	52%	–

Clinical efficacy of entecavir 0.5 mg/day

Switching to entecavir 0.5 mg/day for 1 year resulted in HBV DNA suppression to undetectable levels in the majority of patients with HBV DNA below 5.0 log_{10} copies/mL (100% and 96% for HBV DNA < 2.6 and 2.6–5.0 log_{10} copies/mL, respectively) (Table 3). This proportion was slightly decreased when previous lamivudine treatment duration exceeded 3 years in the 2.6–5.0 log_{10} copies/mL group. In the HBV DNA more than 5.0 log_{10} copies/mL group, approximately half (41%) of the patients achieved viral suppression after 1 year (Table 3); entecavir's efficacy seemed to decrease with prolonged previous exposure to lamivudine, with only 25% of patients having more than 3-year lamivudine treatment achieving undetectable viral load. Similarly, after 2 years, HBV DNA suppression was achieved by 100% and 92% of patients in the HBV DNA less than 2.6 and 2.6–5.0 groups, respectively, and by 44% of patients in the HBV DNA more than 5.0 log_{10} copies/mL group (Table 3).

Among those who failed to suppress viral load, only one case of virological breakthrough was found (2.6–5.0 log_{10} copies/mL group; described under case report). This patient had been previously exposed to lamivudine for more than 3 years.

Alanine aminotransferase levels were normalized in 76–96% and 90–100% of patients following 1 and 2 years of entecavir treatment, respectively (Table 3). HBeAg loss was observed in 27% (3/11), 20% (1/5) and 29% (4/14) of patients with HBV DNA of less than 2.6, 2.6–5.0 and more than 5.0 log_{10} copies/mL, respectively, in the first year.

Lamivudine-resistant substitutions in patients switched to entecavir

Of the 130 patients who received entecavir treatment for at least 1 year, 11 cases failed to suppress HBV DNA to below less than 2.6 log_{10} copies/mL and remained HBV DNA-positive in the first year (1 and 10 in the HBV DNA 2.6–5.0 and > 5.0 log_{10} copies/mL groups, respectively; Table 3). Serum HBV DNA analysis confirmed the presence of rtM204V/I substitutions in 10 of these patients, of which six were rtM204I and three were rtM204V substitutions (Table 4); the remaining patient (2.6–5.0 log_{10} copies/mL group; previous lamivudine exposure 5 years) carried a mixed type substitution, rtM204I plus rtM204V. The only HBV DNA-positive patient who did not

Table 3 Clinical efficacy of entecavir 0.5 mg/day in lamivudine-pretreated patients

End-point by baseline treatment group	Duration of entecavir treatment		
	6 months	1 year	2 years
HBV DNA suppression to undetectable levels, *n*/N (%)			
< 2.6 $\log_{10}$ copies/mL	90/92 (98%)	89/89 (100%)	32/32 (100%)
Previous lamivudine < 1 year	10/10 (100)	9/9 (100)	5/5 (100)
Previous lamivudine 1–3 years	35/35 (100)	35/35 (100)	14/14 (100)
Previous lamivudine > 3 years	45/47 (96)	45/45 (100)	13/13 (100)
2.6–5.0 $\log_{10}$ copies/mL	24/25 (96%)	23/24 (96%)	12/13 (92%)
Previous lamivudine < 1 year	5/5 (100)	5/5 (100)	3/3 (100)
Previous lamivudine 1–3 years	4/4 (100)	4/4 (100)	2/2 (100)
Previous lamivudine > 3 years	15/16 (94)	14/15 (93)	7/8 (88)
> 5.0 $\log_{10}$ copies/mL	5/17 (29%)	7/17 (41%)	4/9 (44%)
Previous lamivudine < 1 year	2/6 (33)	3/6 (50)	2/4 (50)
Previous lamivudine 1–3 years	2/7 (29)	3/7 (43)	2/4 (50)
Previous lamivudine > 3 years	1/4 (25)	1/4 (25)	0/1 (0)
ALT normalization, *n*/*n* (%)			
< 2.6 $\log_{10}$ copies/mL	88/92 (96%)	83/89 (93%)	32/32 (100%)
2.6–5.0 $\log_{10}$ copies/mL	24/25 (96%)	23/24 (96%)	12/13 (92%)
> 5.0 $\log_{10}$ copies/mL	14/17 (82%)	13/17 (76%)	9/10 (90%)

ALT, alanine aminotransferase; HBV, hepatitis B virus.

Table 4 HBV DNA positive rates in patients switched to entecavir 0.5 mg/day for at least 1 year

	HBeAg status	YMDD motif substitution	HBV DNA positive rate, *n*/N (%)	Duration of previous lamivudine treatment, years per patient
Baseline treatment group				
< 2.6 $\log_{10}$ copies/mL	Positive	Wild (or none)	0/10 (0%)	n/a
		YIDD	0/1 (0%)	n/a
	Negative	Wild (or none)	0/58 (0%)	n/a
		YIDD	0/15 (0%)	n/a
		YVDD	0/4 (0%)	n/a
		YIDD + YVDD	0/1 (0%)	n/a
2.6–5.0 $\log_{10}$ copies/mL	Positive	Wild (or none)	0/4 (0%)	
		YIDD + YVDD	1/1 (100%)†	5.0
	Negative	Wild (or none)	0/2 (0%)	n/a
		YIDD	0/10 (0%)	n/a
		YVDD	0/6 (0%)	n/a
		YIDD + YVDD	0/1 (0%)	n/a
> 5.0 $\log_{10}$ copies/mL	Positive	Wild (or none)	1/4 (25%)	0.2
		YIDD	6/9 (67%)	0.5; 1.3; 1.5; 2.7; 3.9; 7.4
		YVDD	1/1 (100%)	0.7
	Negative	YIDD	0/1 (0%)	n/a
		YVDD	2/2 (100%)	1.8; 4.5
All patients			11/130 (8%)	

YMDD motif substitutions: wild, rt204M; YIDD, rt204I; YVDD, rt204V; YIDD + YVDD, rt204I + rt204V.
†Patient with lamivudine-resistant HBV who developed entecavir resistance.
HBeAg, hepatitis B early antigen; HBV, hepatitis B virus; n/a, not available.

carry any detectable lamivudine-resistant substitution had the shortest previous lamivudine exposure (< 6 months; Table 4).

Of the 10 patients carrying rtM204V/I substitutions, eight were HBeAg-positive; the other two patients were HBeAg-negative and carried a lamivudine-resistant rtM204V type substitution.

Emergence of entecavir-resistant mutant: case report

One patient (2.6–5.0 $\log_{10}$ copies/mL group) carrying a mixed substitution YIDD + YVDD (rtM204I + rtM204V) developed entecavir resistance with a recognized rtS202G substitution

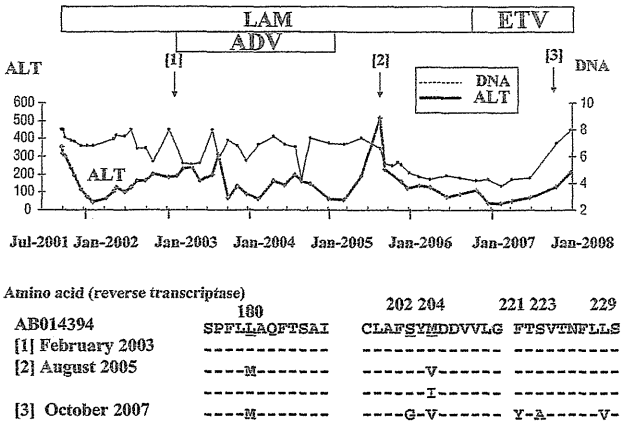

Figure 1 Clinical course and evolution of viral polymerase reverse transcriptase gene sequence in a patient with confirmed rtM204V/I substitutions (YIDD + YVDD) and emerging entecavir resistance substitution (rtS202G). AB014394 was a strain reported by Takahashi *et al.*[33] Two kinds of strains emerged in August 2005 (rtL180M/rtM204V and rtM204I). In October 2007, an additional amino acid substitution (rtS202G) was detected. ADV, adefovir; ALT, alanine aminotransferase; ETV, entecavir; LAM, lamivudine. — DNA; — ALT.

(Table 4). Figure 1 describes the clinical course and evolution of viral DNA sequence. This 37-year-old Japanese man was found to be seropositive for hepatitis B surface antigen with mild ALT elevation in December 1998. He was diagnosed with CHB by peritoneoscopy and liver biopsy (mild hepatitis [A1] and mild fibrosis [F1]). HBeAg was positive; serum HBV DNA was more than 7.6 $\log_{10}$ copies/mL. Treatment with lamivudine 100 mg/day was initiated in October 2001, at which time serum HBV DNA was more than 7.6 $\log_{10}$ copies/mL and ALT was 314 IU/L. In February 2003, adefovir dipivoxil 10 mg/day was added-on to lamivudine, but failed to decrease HBV DNA load. In January 2005, adefovir was withdrawn; the patient remained on lamivudine monotherapy. Amino acid substitutions of the rt gene, rtL180M, rtM204V and rtM204I were detected in August 2005. In October 2006, the patient was switched directly from lamivudine to entecavir 0.5 mg/day without treatment interruption. In February 2007, ALT levels decreased to within normal values, and serum HBV DNA was less than 4 $\log_{10}$ copies /mL. However, shortly after, both ALT levels and HBV DNA began to rise again. In October 2007, amino acid substitutions rtL180M, rtM204V and rtS202G were detected.

Predictive factors of HBV DNA negativity

Univariate analyses identified six factors that correlated with HBV DNA suppression to undetectable levels after 6 months of the entecavir switch: viral load less than 5 $\log_{10}$ copies/mL ($P < 0.001$); HBeAg-negative status ($P < 0.001$); the absence of lamivudine resistance ($P < 0.001$); normal AST level (≤ 33 IU/L; $P = 0.008$); normal ALT level (men ≤ 42 IU/L, women ≤ 27 IU/L; $P < 0.001$); and chronic hepatitis stage of liver disease ($P = 0.069$). Multivariate analyses showed that viral load below 5 $\log_{10}$ copies/mL (OR = 69.03; 95% CI = 13.23–360.09; $P < 0.001$) and the absence of lamivudine resistance (OR = 8.17; 95% CI = 1.25–53.34; $P = 0.028$) each independently influenced entecavir's efficacy to suppress HBV DNA to undetectable levels after 6 months.

Discussion

Entecavir is recommended as a first-line CHB treatment by all major guidelines, due to its antiviral potency and high genetic barrier to resistance in nucleos(t)ide-naïve patients.[2,5,6] Conversely, in lamivudine-resistant patients, switching to entecavir is not a first-choice treatment, due to increased risk of emergence of entecavir resistance on a multiple substitution background.[22,23] However, in attempts to rescue those with suboptimal antiviral response and also to avoid the emergence of viral resistance in responsive patients during their treatment course, switching to entecavir is recommended by the Japanese Ministry of Health, Welfare and Labor for lamivudine-pretreated patients with undetectable viral load (< 2.6 $\log_{10}$ copies/mL), and for patients with detectable HBV DNA but without biochemical breakthrough and lamivudine resistance.[28] This study provides a unique opportunity to evaluate the efficacy of entecavir in a lamivudine-pretreated population with low viral load at switching point.

The majority of patients with HBV DNA at baseline of less than 5 $\log_{10}$ copies/mL maintained or achieved viral suppression 1 year after switching to entecavir, despite 23–76% of them carrying lamivudine-resistant substitutions. A similar trend was maintained during the second year. Conversely, viral suppression below detection limits was reported in less than half of patients with high viral load at baseline (HBV DNA 5.1–7.6 $\log_{10}$ copies/mL) carrying rtM204V/I substitutions (76% patients), in agreement with earlier studies showing diminished entecavir efficacy in lamivudine-refractory patients with elevated viral load.[22,23,29] In addition, multivariate analyses revealed that a viral load of less than 5 $\log_{10}$ copies/mL was an independent predictive factor of HBV DNA suppression to undetectable levels, after 6 months of entecavir therapy. Taken together, these data suggest that switching to entecavir is mostly efficacious in patients with low viral load regardless of the presence of rtM204V/I substitutions. This observation adds another perspective in predicting clinical response to entecavir in lamivudine-pretreated patients.

Another predictive factor of entecavir's efficacy in this retrospective cohort is the absence of lamivudine resistance. This is consistent with previous research suggesting decreased genetic barrier of entecavir to resistance in the presence of lamivudine-resistant substitutions.[22,23] The responsiveness of lamivudine-resistant patients with low viral load reported here could be explained by the ability of entecavir to clear low loads of rtM204V/I mutants. This is suggested by *in vitro* data showing maintained sensitivity of lamivudine-resistant mutants to entecavir, although at higher EC_{50}. Assessing the kinetics of rtM204V/I mutants in response to entecavir switching in patients with undetectable viral load is worth further characterization.

Previous studies have shown that developing entecavir resistance is higher in the presence of pre-existing lamivudine-resistant substitutions.[16–21,30] Despite the presence of lamivudine-resistant virus in 23%–76% of all patient groups, the emergence of entecavir resistance was rare, with only one confirmed case from the

HBV DNA 2.6–5.0 $\log_{10}$ copies/mL group. This patient's history is suggestive of a typical refractory case, with failure of multiple regimens including the combination of lamivudine plus adefovir (Fig. 1). The low entecavir resistance rate in this study may be due to the relatively short treatment period and small sample size. Further follow up will be required to monitor for subsequent emergence of entecavir resistance in these patients.

One could argue whether it is cost-effective to switch all lamivudine-treated patients with undetectable HBV DNA to entecavir. The GLOBE study demonstrated that although fewer lamivudine-treated patients with undetectable HBV DNA at week 24 developed viral resistance, resistance could still occur after 2 years of treatment (9% and 5% of HBeAg-positive and HBeAg-negative patients, respectively).[31] Moreover, Yuen and collaborators also reported that of lamivudine-treated patients who achieved HBV DNA suppression below 200 copies/mL at week 24, 8.3% developed resistance after 5 years.[32] In countries where medicine access is an issue, further studies are needed to evaluate the cost-effectiveness of entecavir switching of all patients with undetectable viral load, versus switching only those at risk of developing viral resistance. Comparative studies integrating the efficacy and safety of standard adefovir add-on versus switching to entecavir monotherapy are also warranted in these patients.

Study limitations should be considered. This is a retrospective analysis of CHB patients which, in the absence of matching controls, may introduce confounding errors and bias. Specifically, a control arm for the HBV PCR-negative group (< 2.6 $\log_{10}$ copies/mL; $n = 92$) would be required to strengthen study conclusions. Another limitation is the small sample size of the intermediate and high HBV DNA cohorts (25 patients with 2.6–5.0 $\log_{10}$ copies/mL, and 17 patients with > 5.0 $\log_{10}$ copies/mL, respectively); adding more patients to these samples as available would add weight to describing higher number entecavir response and resistance rates in these groups.

In conclusion, this study shows that the efficacy of switching from lamivudine to entecavir 0.5 mg/day is highest for Japanese patients with no rtM204V/I substitutions and a viral load of less than 5 $\log_{10}$ copies/mL, independent of their previous exposure to lamivudine. Efficacy is decreased for patients with rtM204V/I substitutions and low viral load, and is lowest for patients with rtM204V/I substitutions and high viral load. Viral resistance to entecavir after 48 weeks is rare in these patients. Multivariate analyses showed that viral load of less than 5 $\log_{10}$ copies/mL and the absence of lamivudine resistance are independent factors predicting entecavir's efficacy to reduce HBV DNA to undetectable levels after 6 months of treatment.

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References

1 Lavanchy D. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. *J. Viral Hepat.* 2004; **11**: 97–107.
2 Liaw Y-F, Leung N, Kao J-H *et al.* Asian-Pacifc consensus statement on the management of chronic hepatitis B: a 2008 update. *Hepatol. Int.* 2008; **2**: 263–83.
3 Chen CJ, Yang HI, Su J *et al.* Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65–73.
4 Iloeje U, Yang HI, Su J, Jen CL, You SL, Chen CJ. Predicting liver cirrhosis risk based on the level of circulating hepatitis B viral load. *J. Gastroenterol.* 2006; 678–86.
5 Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
6 European Association for the Study of the Liver. EASL clinical practice guidelines: management of chronic hepatitis B. *J. Hepatol.* 2009; **50**: 227–42.
7 Locarnini S, Mason WS. Cellular and virological mechanisms of HBV drug resistance. *J. Hepatol.* 2006; **44**: 422–31.
8 Lok AS, Zoulim F, Locarnini S *et al.* Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007; **46**: 254–65.
9 Suzuki F, Tsubota A, Arase Y *et al.* Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection in Japan. *Intervirology* 2003; **46**: 182–9.
10 Chang TT, Gish RG, de Man R *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N. Engl. J. Med.* 2006; **354**: 1001–10.
11 Gish RG, Lok AS, Chang TT *et al.* Entecavir therapy for up to 96 weeks in patients with HBeAg-positive chronic hepatitis B. *Gastroenterology* 2007; **133**: 1437–44.
12 Lai CL, Shouval D, Lok AS *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N. Engl. J. Med.* 2006; **354**: 1011–20.
13 Ren FY, Piao DM, Piao XX. A one-year trial of entecavir treatment in patients with HBeAg-positive chronic hepatitis B. *World J. Gastroenterol.* 2007; **13**: 4264–7.
14 Schiff E, Simsek H, Lee WM *et al.* Efficacy and safety of entecavir in patients with chronic hepatitis B and advanced hepatic fibrosis or cirrhosis. *Am. J. Gastroenterol.* 2008; **103**: 1–8.
15 Colonno RJ, Rose R, Baldick CJ *et al.* Entecavir resistance is rare in nucleoside naive patients with hepatitis B. *Hepatology* 2006; **44**: 1656–65.
16 Tenney DJ, Rose RE, Baldick CJ, Pokornowski K, Eggers BJ. Long-term monitoring shows hepatitis B virus resistance to entecavir in nucleoside-naïve patients is rare through 5 years of therapy. *Hepatology* 2009; **49**: 1503–14.
17 Baldick CJ, Eggers BJ, Fang J *et al.* Hepatitis B virus quasispecies susceptibility to entecavir confirms the relationship between genotypic resistance and patient virologic response. *J. Hepatol.* 2008; **48**: 895–902.
18 Tenney DJ, Rose RE, Baldick CJ *et al.* Two-year assessment of entecavir resistance in Lamivudine-refractory hepatitis B virus patients reveals different clinical outcomes depending on the resistance substitutions present. *Antimicrob. Agents Chemother.* 2007; **51**: 902–11.
19 Tenney DJ, Levine SM, Rose RE *et al.* Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob. Agents Chemother.* 2004; **48**: 3498–507.
20 Nagasaki F, Niitsuma H, Ueno Y *et al.* The high incidence of the emergence of entecavir-resistant mutants among patients infected with lamivudine-resistant hepatitis B virus. *Tohoku J. Exp. Med.* 2007; **213**: 181–6.
21 Villet S, Ollivet A, Pichoud C *et al.* Stepwise process for the development of entecavir resistance in a chronic hepatitis B virus infected patient. *J. Hepatol.* 2007; **46**: 531–8.

22 Sherman M, Yurdaydin C, Simsek H *et al.* Entecavir therapy for lamivudine-refractory chronic hepatitis B: improved virologic, biochemical, and serology outcomes through 96 weeks. *Hepatology* 2008; **48**: 99–108.
23 Sherman M, Yurdaydin C, Sollano J *et al.* Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. *Gastroenterology* 2006; **130**: 2039–49.
24 Suzuki F, Toyoda J, Katano Y *et al.* Efficacy and safety of entecavir in lamivudine-refractory patients with chronic hepatitis B: randomized controlled trial in Japanese patients. *J. Gastroenterol. Hepatol.* 2008; **23**: 1320–6.
25 Matsuyama K, Hayashi K, Miura T. The quantitative assay for HBV-DNA and the detection of HBV-DNA point mutation by polymerase chain reaction—'AMPLICOR HBV MONITOR Test' and 'HBV pre Core/Core Promoter Mutation Detection kit. *Kan Tan Sui* 2000; **41**: 59–71.
26 Kobayashi S, Shimada K, Suzuki H, Tanikawa K, Sata M. Development of a new method for detecting a mutation in the gene encoding hepatitis B virus reverse transcriptase active site (YMDD motif). *Hepatol. Res.* 2000; **17**: 31–42.
27 Suzuki F, Kumada H, Nakamura H. Changes in viral loads of lamivudine-resistant mutants and evolution of HBV sequences during adefovir dipivoxil therapy. *J. Med. Virol.* 2006; **78**: 1025–34.
28 Kumada H. *Scientific Research Grant of Ministry of Health, Labour and Welfare. Research of hepatitis overcome urgent strategy. Research report of the standardization of viral hepatitis treatment including liver cirrhosis* (Japanese version). 2009.
29 Chang TT, Gish RG, Hadziyannis SJ *et al.* A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198–209.
30 Kobashi H, Fujioka S-I, Kumada H, Yokosuka O, Hayashi N, Suzuki K. Emergence of hepatitis B virus gene mutation related to entecavir-resistance in chronic hepatitis B patients participated in the phase 2 clinical studies of entecavir in Japan. *Hepatology* 2007; **46** (Suppl. 1): 666A.
31 Lai CL, Gane E, Liaw YF *et al.* Telbivudine versus lamivudine in patients with chronic hepatitis B. *Hepatology* 2006; **44**: 222A.
32 Yuen MF, Fong DY, Wong DK, Yuen JC, Fung J, Lai CL. Hepatitis B virus DNA levels at week 4 of lamivudine treatment predict the 5-year ideal response. *Hepatology* 2007; **46**: 1695–703.
33 Takahashi K, Akahane Y, Hino K, Ohta Y, Mishiro S. Hepatitis B virus genomic sequence in the circulation of hepatocellular carcinoma patients: comparative analysis of 40 full-length isolates. *Arch Virol* 1998; **143**: 2312–26.

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CLINICAL STUDIES

HBcrAg is a predictor of post-treatment recurrence of hepatocellular carcinoma during antiviral therapy

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covalently closed circular DNA – HBcrAg – HCC recurrence – nucleot(s)ide analogue – portal vein invasion

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Abstract

Background/Aims: The recurrence rate of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC) is high even in patients receiving curative therapy. In this study, we analysed the risk factors for tumour recurrence after curative therapy for HBV-related HCC while under treatment with nucleot(s)ide analogues (NAs) by measuring serum HBcrAg and intrahepatic covalently closed circular DNA (cccDNA) levels to elucidate the viral status associated with HCC recurrence. *Methods:* We enrolled 55 patients who developed HCC during NA therapy and underwent either curative resection or percutaneous ablation for HCC. *Results:* Hepatocellular carcinoma recurred in 21 (38%) of the patients over a period of 2.2 (range, 0.2–7.4) years. In multivariate analysis, serum HBcrAg levels $\geq$4.8log U/ml at the time of HCC diagnosis (hazard ratio, 8.96; 95% confidential interval, 1.94–41.4) and portal vein invasion (3.94, 1.25–12.4) were independent factors for HCC recurrence. The recurrence-free survival rates of the high cccDNA group were significantly lower than those of the low cccDNA group only in patients who underwent resection ($P = 0.0438$). A positive correlation ($P = 0.028$; $r = 0.479$) was observed between the intrahepatic cccDNA and the serum HBcrAg levels at the incidence of HCC. *Conclusion:* HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Serum HBcrAg and intrahepatic cccDNA suppression by NAs may be important to prevent HCC recurrence.

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and one million people die of decompensated cirrhosis and/or hepatocellular carcinoma (HCC) annually (1, 2). Recently, oral nucleot(s)ide analogues (NAs) have been used as the mainstay therapeutic strategy against chronic hepatitis B. Five such antiviral agents have been approved, and range in the profundity and rapidity of HBV DNA suppression, barrier to resistance and side-effect profile (3–10). Lamivudine (LAM) was the first NA to be approved for treating chronic hepatitis B, followed by adefovir dipivoxil (ADV) and entecavir (ETV), in Japan. However, a major problem with long-term LAM treatment is the potential development of drug resistance, mainly caused by mutation of the thyrosine–methionine–aspartic acid–aspartic acid (YMDD) motif of reverse transcriptase (11, 12). For preventing breakthrough hepatitis induced by LAM-resistant mutants, additional ADV administration has been recommended (13, 14).

The methods for monitoring the treatment response include measurements of the serum alanine transaminase (ALT) levels, HBV DNA levels, HBeAg and antibody levels, HBsAg and antibody levels and liver histology. Other serum markers have been reported to be useful for monitoring the effect of antiviral therapy (15, 16). Recently, a new assay was developed for detecting the HBcrAg, consisting of HBcAg, HBeAg and a 22 kDa precore protein coded with the precore/core gene (17, 18). Because NAs have no inhibiting action on the transcription and translation activities of viral mRNA, HBcAg- and HBeAg-related proteins continue to be produced for a certain period of time in spite of the achievement of adequate suppression of the viral DNA synthesis. Therefore, HBcrAg is a viral marker independent of HBV DNA for monitoring the antiviral effect of NAs (19). In addition, recent reports have indicated another interesting aspect of serum HBcrAg levels: these levels were found to be correlated with intrahepatic covalently closed circular DNA (cccDNA) levels and could be a surrogate marker of the intrahepatic cccDNA pool (20, 21). This phenomenon may be explained by the fact that the production of HBcrAg depends on the

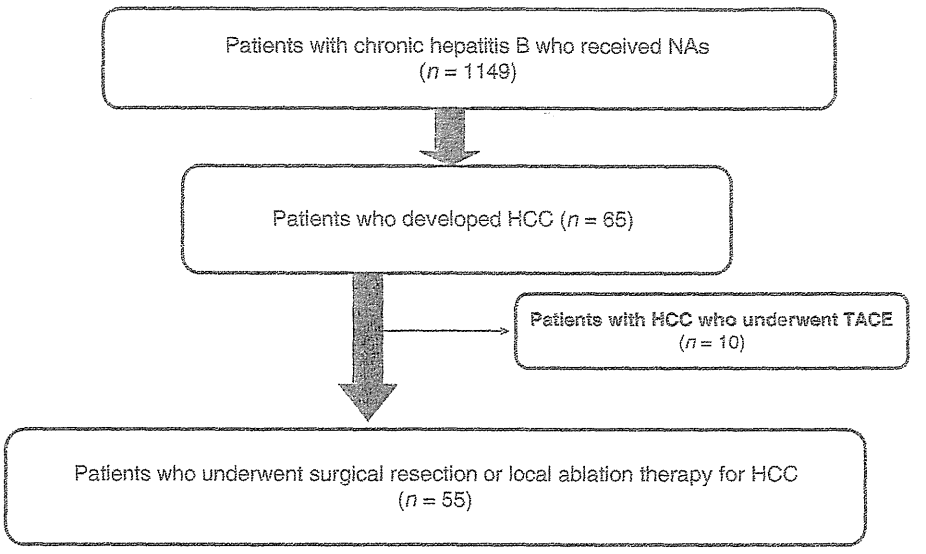

Fig. 1. The study protocol. HCC, hepatocellular carcinoma; NAs, nucleot(s)ide analogues; TACE, transcatheter arterial chemoembolization.

transcription of mRNA from cccDNA, and that cccDNA still remains in high levels during treatment with NAs.

Although patients with HBV-related cirrhosis have a significantly high risk of developing HCC, NA therapy can delay the progression of liver disease and reduce the risk of HCC in patients with cirrhosis by strong viral suppression (22, 23). Nevertheless, a few cases develop HCC during NA therapy at a constant rate (3–12%) (22, 24–26). The recurrence rate of HBV-related HCC after curative resection is estimated to be high, and is associated with viral factors, including HBeAg positivity and the viral load before surgery, besides host and tumour factors, but these findings were demonstrated in the absence of antiviral therapy (27–30). However, almost all patients, receiving NAs, showed negativity of serum HBV DNA. And so, we made the hypothesis that intrahepatic viral status, such as intrahepatic cccDNA and serum HBcrAg levels of its surrogate maker, might have an impact on tumour recurrence during NA therapy.

In this study, we examined the risk factors for tumour recurrence after curative resection and ablation for HBV-related HCC during NA therapy by measuring the serum HBcrAg and intrahepatic cccDNA levels with the aim to elucidate the viral status, persistent despite suppressive therapy, associated with HCC recurrence, in addition to the host and tumour factors reported in the past.

Patients and methods

Patients

Over a period of 13 years, from September 1995 to September 2008, 1149 patients with chronic hepatitis B received NA therapy, including LAM, ADV and ETV, at the Department of Hepatology, Toranomon Hospital, Metropolitan Tokyo. Of the 1149 patients, 65 developed HCC after the start of NA therapy from February 2001 to June 2009. Of the 65 consecutive patients, 55 underwent radical therapy, including either resection or percutaneous ablation as the initial therapy for HCC. These 55 patients were enrolled in this cohort study (Fig. 1). The median duration from the start of NA therapy to the development of HCC was 2.2 (range, 0.2–7.4) years. The exclusion criteria were (i) patients co-infected with hepatitis C, delta or human immunodeficiency virus and (ii) a history of other liver diseases such as autoimmune hepatitis, alcoholic liver disease or metabolic liver disease.

The diagnosis of HCC was predominantly based on imaging, including dynamic computed tomography, magnetic resonance imaging and/or digital subtraction angiography. When the hepatic nodule did not show the typical imaging features, fine needle aspiration biopsy was performed, followed by histological examination and diagnosis. The physicians and surgeons usually discussed the preferred choice of treatment for each patient. Hepatic resection was mainly performed for patients categorized as Child–Pugh grade A or B liver function, and had no serious complications. Percutaneous ablation was performed for patients with surgical contraindications or for those who did not prefer to undergo hepatic resection by using two different devices: the cool-tip system (Tyco Healthcare Group LP, Burlington, VT, USA) and the radiofrequency tumour coagulation system (RTC system; Boston-Scientific Japan Co., Tokyo, Japan). The term curative treatment was used to indicate that no tumours were left in the remnant liver, irrespective of the width of the margin around the tumour, confirmed using intra-operative ultrasonography, combined ultrasonography and dynamic computed tomography 1 month after the resection or ablation. Serum samples were collected from all patients before and after

the treatment for HCC and stored in −80 °C. Liver tissue from patients who underwent resection was collected, rapidly frozen and stored in −80 °C. Written informed consent was obtained from each patient. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki, as reflected in *a priori* approval by the institution's human research committee.

Antiviral therapy

Forty-seven patients received 100 mg LAM daily, and drug-resistant YMDD mutants developed in 26 (55%) of these patients, accompanied by an increase in HBV DNA ≥1 log copies/ml. Seventeen of the 26 patients received 10 mg ADV in addition to LAM (100 mg) daily. The remaining nine continued to receive LAM monotherapy because of the lack of approval for ADV administration in Japan at the time, but received ADV with LAM after approval was obtained during the HCC posttreatment period. Eight NA-naïve patients received 0.5 mg ETV daily. These antiviral therapies were continued after the resection or percutaneous ablation.

Follow-up and HCC recurrence

The patients were followed for liver function and virological markers of HBV infection monthly, as well as blood counts and tumour makers including α-fetoprotein and des-γ-carboxylprothrombin. They also underwent ultrasonography or helical dynamic computed tomography every 3 months. Cirrhosis was diagnosed by laparoscopy or liver biopsy or by the clinical data, imaging modalities and portal hypertension. The median observation period after HCC treatment for the entire cohort was 2.7 years (range, 0.3–8.4 years). HCC recurrence was diagnosed by the typical hypervascular characteristics on angiography and/or histological examination with fine needle biopsy specimens, in addition to certain features on computed tomography and ultrasonography.

Markers of HBV infection

HBeAg was determined by enzyme-linked immunosorbent assay using a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). HBV DNA was quantitated using the Amplicor monitor assay (Roche Diagnostics, Tokyo, Japan) with a dynamic range over 2.6–7.6log copies/ml or COBAS TaqMan HBV v.2.0 (Roche Diagnostics) with a dynamic range over 2.1–9.0log copies/ml. Serum HBV DNA levels were measured using the Amplicor assay at both the start of NA therapy and the diagnosis of HCC and using the TaqMan assay at the diagnosis of HCC. For statistical analysis, the value of that HBV DNA was tentatively set at 2.1 if HBV DNA levels were under 2.1log copies/ml. HBV genotypes were determined serologically by the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the seven major genotypes (A–G), using a commercial kit (HBV Genotype EIA; Institute of Immunology). YMDD mutants were determined by polymerase chain reaction-based enzyme-linked minisequence assay using a commercial kit (Genome Science Laboratories, Tokyo, Japan).

HBcrAg measurement

Serum HBcrAg levels were measured using a CLEIA HBcrAg assay kit (Fujirebio Inc., Tokyo, Japan) with a fully automated analyser system (Lumipulse System; Fujirebio Inc.) as described previously (21). In brief, 150 μl of serum was incubated with 150 μl of pretreatment solution containing 15% sodium dodecyl sulphate at 60 °C for 30 min. After heat treatment, 120 μl of pretreated specimen was added to a ferrite microparticle suspension in an assay cartridge. Ferrite particles were coated with monoclonal antibody mixture (HB44, HB61 and HB114) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After 10 min of incubation at 37 °C and washing, further incubation was carried out for 10 min at 37 °C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg and the 22 kDa precore protein. After washing, 200 μl of substrate solution [3-(2′-spiroada-mantan)-4-methoxy-4-(3′-phosphoryloxy)pheny1-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA, USA) was added to the test cartridge, which was then incubated for 5 min at 37 °C. The relative chemiluminescence intensity was measured, and the HBcrAg concentration was calculated by a standard curve generated using a recombinant pro-HBeAg (amino acids −10 to 183 of the precore/core gene product). The HBcrAg concentration was expressed in U/ml, which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, the HBcrAg values were expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analyses, HBcrAg-negative cases were calculated as 3.0log U/ml.

Intrahepatic cccDNA measurement

Intrahepatic cccDNA levels were analysed as described previously (21). In brief, liver specimens surrounding the tumour tissue were obtained and stored at −80 °C before DNA extraction. HBV DNA was extracted using a QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on the absorbance at 260 nm. For this study, two oligonucleotide primers cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1424–1444) and cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1755–1737) and probe cccP2 (5′-VIC-accaatttat gcctacag-MGB-3′, nucleotides 1672–1655) were designed using PRIMER EXPRESS software (Applied Biosystems, Foster City, CA, USA) to flank the direct repeat region between the hepatitis B core and the polymerase gene. The use of cccF2 and cccR4, oligonucleotide primers spanning the direct repeat region of the HBV genome, allows the polymerase chain reaction of native viral DNA in the

Dane particle to block the amplification of products, because the partially double-stranded HBV DNA is disrupted in the direct repeat region. Twenty-five microlitres of extracted DNA (0.5 μg) was detected with the sequence detector system (ABI 7900HT; Applied Biosystems) in 50 μl of a PCR mixture containing TaqMan universal PCR Master Mix (Applied Biosystems), 300 nmol of each primer and 250 nmol of the probe. After initial activation of uracil-*N*-glycosylase at 50 °C for 2 min, AmpliTaq Gold (Applied Biosystems) was activated at 95 °C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95 °C for 15 s, and annealing and extension at 60 °C for 90 s per cycle (SRL Inc., Tokyo, Japan).

Statistical analyses

Standard statistical measures and procedures were used. Correlations between two variables were tested using Pearson's correlation analysis. Cox regression analysis was used to assess significant associations of the risk factors with tumour recurrence after HCC treatment. All factors found to be at least associated with recurrence ($P < 0.05$) were tested by multivariate analysis. Independent factors, associated with HCC recurrence, were calculated using stepwise Cox regression analysis. The cumulative recurrence-free survival rates after HCC treatment were analysed using the Kaplan–Meier method, and differences in the curves were tested using the log-rank test. A P value of < 0.05 in a two-tailed test was considered significant. Data analysis was performed with SPSS version 11.0 (SPSS Inc., Chicago, IL, USA).

Results

Patient characteristics at the start of NA therapy and HCC incidence

Table 1 presents a comparison of the patient characteristics at the start of NA therapy and the time of HCC diagnosis. Almost all the patients (93%) enrolled in this study had HBV genotype C. One patient had genotype B, and the genotypes of three patients could not be determined. The rate of HBV DNA disappearance from serum in all the patients was 64% (35/55; Amplicor monitor assay, < 2.6log copies/ml) and 51% (28/55; TaqMan assay, < 2.1log copies/ml), that of aspartate aminotransferase (AST) normalization (< 32 IU/L) was 56% (31/55) and that of ALT normalization (< 42 IU/L) was 71% (39/55) at the incidence of HCC. YMDD mutants were detected in 30 of 47 patients at the beginning of LAM monotherapy, and virological breakthrough (VBT), accompanied by an increase in HBV DNA (≥ 1log copies/ml), occurred in 26 patients with YMDD mutants by the diagnosis of HCC. Seventeen of these patients received ADV with LAM. No resistant mutation to ADV (rtA181T/S, rtN236T) occurred in patients receiving the combination therapy. Further, no drug-resistant mutant

Table 1. Patient characteristics at the start of nucleot(s)ide analogue therapy and the incidence of hepatocellular carcinoma

Characteristics	Start of NA therapy	Time of HCC Dx
Age (years)	51 (32–73)	54 (35–75)
Gender (male:female)	45:10	45:10
AST level (IU/L)	69 (27–195)	31 (16–207)
ALT level (IU/L)	78 (23–368)	29 (10–267)
Platelet count (10^5/mm^3)	11.4 (3.1–31.3)	12.9 (3.6–30.1)
Serum albumin level (g/dl)		3.8 (3.1–4.4)
Serum bilirubin level (mg/dl)		0.9 (0.4–2.4)
Prothrombin time (%)		90.8 (59–112)
Indocyanine green retention rate at 15 min (%)		14.5 (4–53)
Child–Pugh (A:B)		49:6
HBV genotype		
C	51 (93%)	51 (93%)
Others	4	4
HBeAg (+)	29 (53%)	23 (42%)
HBV DNA (log copies/ml)	7.1 (< 2.6 to > 7.6)	< 2.1 (< 2.1 to 8.5)
HBcrAg level (log U/ml)	6.6 (3.3 to > 6.8)	5.0 (< 3.0 to > 6.8)
Antiviral agents (LAM:LAM+ADV:ETV)	47:0:8	30:17:8
Duration of NA therapy before the incidence of HCC (years)		2.2 (0.2–7.4)
α-fetoprotein level (ng/dl)	6 (2–263)	4 (1–282)
Des-γ-carboxylprothrombin level (mAU/ml)		22 (< 10–933)
Tumour diameter (mm)		22 (7–60)
Tumour number (solitary:multiple)		50:5
Portal vein invasion (positive:negative)		49:6
TNM stage (I:II:III:IV)		25: 24: 5: 1
HCC treatment (resection:ablation)		37:18

Values are expressed as the median and range (parenthetically) or the number and percentage (parenthetically).
ADV, adefovir dipivoxil; ETV, entecavir; HBV DNA, hepatitis B virus DNA; HCC, hepatocellular carcinoma; LAM, lamivudine; NA, nucleot(s)ide analogues.

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was detected in the NA-naïve patients receiving ETV monotherapy.

Correlation between serum HBcrAg and serum HBV DNA levels at the incidence of HCC

The median serum HBcrAg value was 6.6log U/ml (range, 3.3 to > 6.8) at the start of NA therapy and 5.0log U/ml (range, < 3.0 to > 6.8) at the time of HCC diagnosis. We observed a positive correlation ($P < 0.001$; $r = 0.610$) between the levels of HBcrAg and HBV DNA in serum at the time of HCC diagnosis (Fig. 2A).

HBcrAg was detectable in 23 (82%) of 28 patients with undetectable HBV DNA levels using TaqMan assay and was > 4.8log U/ml in eight (29%) of 28 patients. In contrast, serum HBV DNA was detectable in spite of undetected HBcrAg in only two patients. Then, we examined the correlation between the serum HBcrAg levels at the time of HCC diagnosis and the antiviral effect. The median duration of on-treatment undetected serum HBV DNA was 1.1 years (range, 0.1–4.8) before the first diagnosis of HCC. As shown in Figure 2B, we observed a significant negative correlation between the levels of HBcrAg in serum at the time of HCC diagnosis and the duration of undetected HBV DNA in serum just before the first diagnosis of HCC ($P < 0.001$; $r = -0.568$).

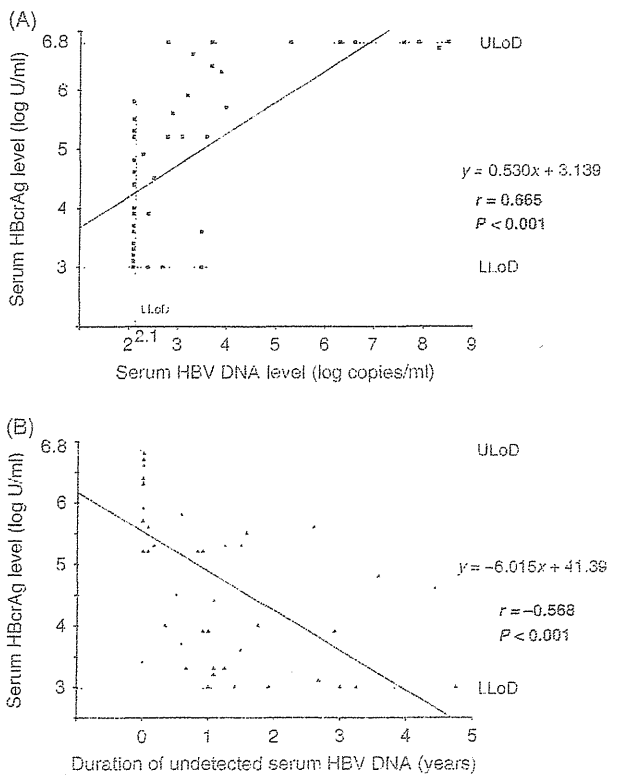

Fig. 2. (A) Correlation between serum HBcrAg and hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient. (B) Correlation between serum HBcrAg levels at the time of HCC diagnosis and the duration of undetected serum HBV DNA (< 2.6log copies/ml).

Factors associated with HCC recurrence

Hepatocellular carcinoma recurred in 21 (38%) of the 55 patients, 17 (46%) of 37 patients who had undergone resection and four (22%) of 18 patients who had undergone ablation. Because a proportion of patients who had undergone resection with TNM Stage II or over (24 of 37 patients) was greater than ablation (six of 18), there were more patients who had HCC recurrence after resection than ablation. Eight factors were associated with the recurrence in univariate analysis: HBeAg positivity at the start of NA therapy, HBV DNA ≥ 2.1log copies/ml, HBcrAg level ≥ 4.8log U/ml, AST level ≥ 50 IU/L, ALT level ≥ 40 IU/L, tumour multiplicity, portal vein invasion at the time of HCC diagnosis and HCC treatment. In the multivariate analysis, HBcrAg level ≥ 4.8log U/ml and portal vein invasion were independent risk factors for the recurrence of HCC (Table 2). The cumulative recurrence-free survival rates in patients with ≥ 4.8log U/ml HBcrAg levels at the time of HCC diagnosis were 70% at 1 year, 35% at 3 years and 28% at 5 years. In contrast, the rates in patients with < 4.8log U/ml HBcrAg levels were 96% at 1 year, 89% at 3 years and 89% at 5 years. The recurrence-free survival rates of the high HBcrAg group (≥ 4.8log U/ml) were significantly lower than those of the low HBcrAg group (< 4.8log U/ml; $P < 0.001$), as shown in Figure 3A. Then, the cumulative recurrence-free survival rates in patients with ≥ 2.1log copies/ml HBV DNA levels at the time of HCC diagnosis were 70% at 1 year, 44% at 3 years and 39% at 5 years. In contrast, the rates in patients with < 2.1log copies/ml HBV DNA levels were 93% at 1 year, 76% at 3 years and 76% at 5 years. The recurrence-free survival rates of the positive HBV DNA group (≥ 2.1log copies/ml) were significantly lower than those of the negative HBV DNA group (< 2.1log copies/ml; $P = 0.007$), as shown in Figure 3B. The cumulative recurrence-free survival rates were 33% at 1 year and 33% at 2 years with portal vein invasion, and 87% at 1 year, 73% at 2 years and 64% at 3 years without invasion. Three of the six patients with portal vein invasion died of recurrent HCC.

Correlation between intrahepatic cccDNA and serum HBV DNA levels at the incidence of HCC

We measured intrahepatic cccDNA using liver specimens from 22 of 37 patients who underwent resection. The median intrahepatic cccDNA value was 4.2log copies/μg (range, 3.0–5.0). As shown in Figure 4A and B, we observed significant positive correlations between the levels of intrahepatic cccDNA and HBV DNA in serum ($P = 0.019$; $r = 0.486$) and between the levels of intrahepatic cccDNA and HBcrAg in serum at the time of HCC diagnosis ($P = 0.028$; $r = 0.479$). Twenty-eight patients who underwent resection had early- or intermediate-stage

Table 2. Risk factors for hepatocellular carcinoma recurrence

	Univariate analysis		Multivariate analysis	
Factors	Hazard ratio (95% CI)	*P*	Hazard ratio (95% CI)	*P*
Start of NA therapy				
Age (≥50 years)	1.79 (0.65–4.91)	0.257		
Gender (female)	0.98 (0.32–2.97)	0.981		
HBeAg(+)	2.85 (1.03–7.88)	**0.044**		
HBV DNA (≥6.0log copies/ml)	1.75 (0.50–6.07)	0.378		
AST level (≥50 IU/L)	1.09 (0.42–2.85)	0.862		
ALT level (≥70 IU/L)	1.09 (0.42–2.85)	0.862		
Platelet count (< 1.2×10^5 cells/mm^3)	2.56 (0.96–6.85)	0.061		
α-fetoprotein level (≥100 ng/ml)	0.99 (0.13–7.66)	0.996		
Time of HCC diagnosis				
Duration of NA therapy (≥2 years)	1.19 (0.49–2.88)	0.698		
HBeAg(+)	1.53 (0.63–3.70)	0.343		
HBV DNA (≥2.1log copies/ml)	3.36 (1.32–8.55)	**0.011**		
HBcrAg level (≥4.8log U/ml)	10.6 (2.45–46.1)	**0.002**	**8.96 (1.94–41.4)**	**0.005**
YMDD mutants (present:absent)	0.84 (0.35–2.03)	0.838		
AST level (≥50 IU/L)	2.44 (1.01–5.89)	**0.047**		
ALT level (≥40 IU/L)	2.44 (1.01–5.87)	**0.047**		
Platelet count (< 10^5 cells/mm^3)	2.20 (0.81–6.02)	0.123		
Serum albumin level (< 3.5 g/dl)	1.39 (0.53–3.63)	0.505		
Serum bilirubin level (≥1.5 mg/dl)	1.11 (0.62–2.00)	0.713		
Prothrombin time (< 80%)	2.23 (0.51–9.82)	0.286		
Child–Pugh (B)	0.70 (0.16–3.04)	0.634		
Indocyanine green retention rate at 15 min (≥30%)	0.58 (0.17–1.99)	0.389		
α-fetoprotein level (≥100 ng/ml)	1.81 (0.74–4.44)	0.194		
Des-γ-carboxylprothrombin level (≥100 mAU/ml)	2.09 (0.81–5.39)	0.129		
Tumour size (≥21 mm)	2.02 (0.81–5.07)	0.133		
Tumour number (multiple)	3.94 (1.29–12.1)	**0.016**		
Portal vein invasion	5.39 (1.69–17.2)	**0.004**	**3.94 (1.25–12.4)**	**0.019**
TNM stage (≥II)	2.08 (0.85–5.10)	0.110		
HCC treatment (resection)	3.10 (1.05–9.09)	**0.041**		

The bolded numbers: statically significant.

ALT, alanine transaminase; AST, aspartate aminotransferase; CI, confidence interval; HBV DNA, hepatitis B virus DNA; NA, nucleot(s)ide analogues; YMDD, thyrosine–methionine–aspartic acid–aspartic acid.

HCC (tumour diameter < 50 mm, absence of vascular invasion and well/moderately differentiated). In 17 of these patients, the intrahepatic cccDNA levels were measured using the resected specimens. The recurrence-free survival rates of the high cccDNA group (≥4.3log copies/μg) were significantly lower than those of the low cccDNA group (< 4.3log copies/μg; $P = 0.0438$), as shown in Figure 4C.

Comparison of the serum HBcrAg levels and the patient characteristics

We examined whether the serum HBcrAg levels at the time of HCC diagnosis were correlated with the baseline parameters before antiviral therapy. The HBcrAg levels were compared with the baseline HBeAg-positive and HBeAg-negative status and with the baseline HBV DNA levels ≥6.0log and < 6.0log copies/ml (Fig. 5). The HBcrAg levels were significantly higher in patients who were positive for HBeAg (median value: 5.6 vs. 3.6log U/ml; $P = 0.001$) and the baseline HBV DNA levels ≥6.0log copies/ml (median value: 5.2 vs. 3.3log U/ml; $P = 0.012$). There was no correlation between the other baseline parameters at the start of NA therapy and the serum HBcrAg levels at the time of HCC diagnosis. Then, we examined whether the serum HBcrAg levels at the time of HCC diagnosis were associated with on-treatment drug resistance during antiviral therapy. Figure 6 shows the comparison of the serum HBcrAg levels at the time of HCC diagnosis with or without the emergence of YMDD mutants and VBT before the development of HCC. The HBcrAg levels were marginally higher in patients with emergent YMDD mutants (median value: 5.2 vs. 3.8log U/ml; $P = 0.051$) and significantly higher in those with VBT (median value: 5.2 vs. 3.9log U/ml; $P = 0.006$). There was no correlation between serum HBcrAg at the time of HCC diagnosis and age of patients or tumour factors.

Discussion

In this study, we examined whether the intrahepatic cccDNA and HBcrAg levels as substitutes for cccDNA are associated with HCC recurrence in patients who

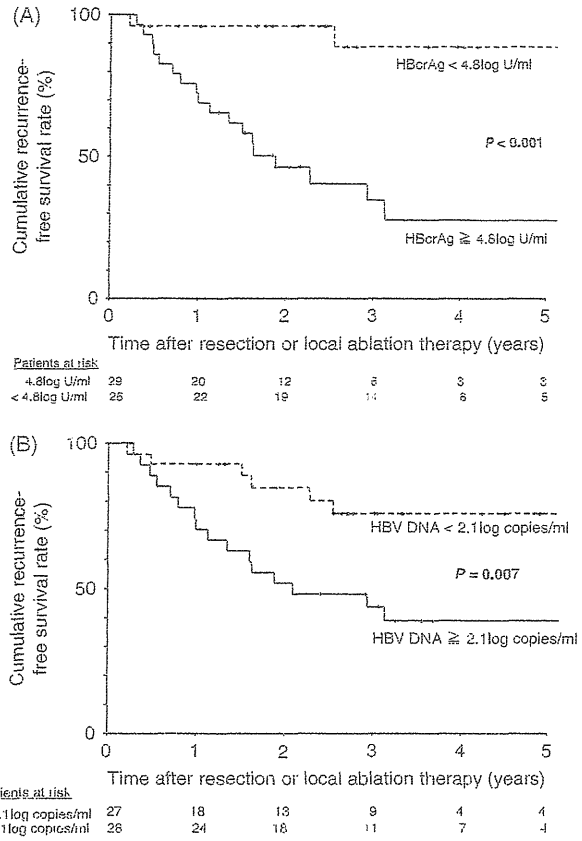

Fig. 3. (A) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum HBcrAg levels and comparison by the log-rank test. (B) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient and comparison by the log-rank test.

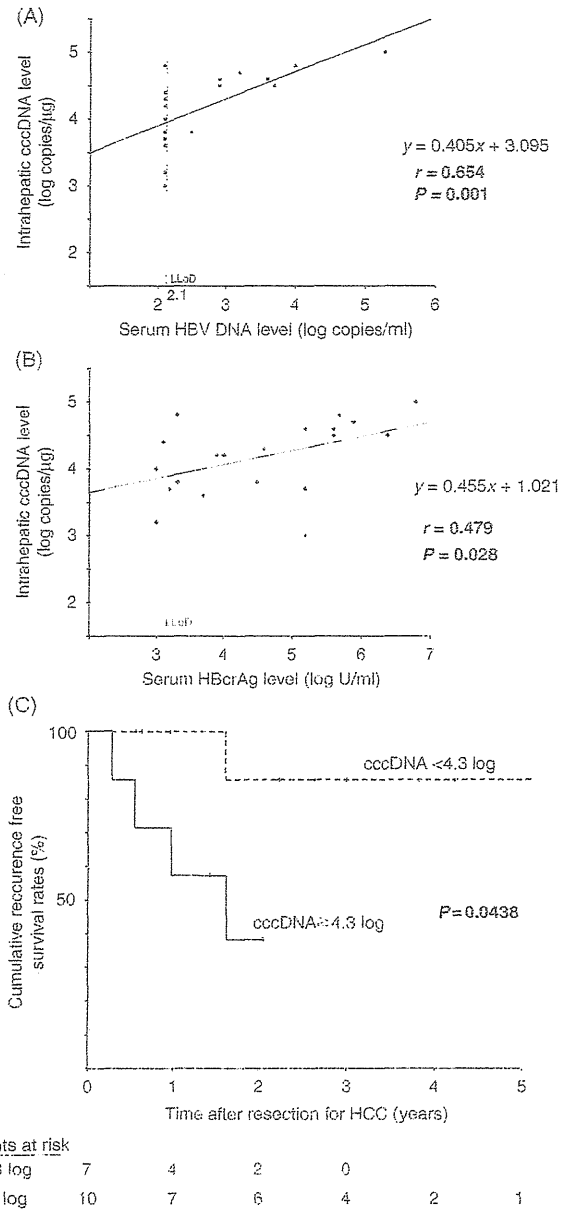

Fig. 4. (A) Correlation between intrahepatic covalently closed circular DNA (cccDNA) and serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient who underwent resection ($n = 22$). (B) Correlation between intrahepatic cccDNA and serum HBcrAg levels at the time of HCC diagnosis. (C) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the intrahepatic cccDNA levels in patients with early- or intermediate-stage HCC ($n = 17$).

developed HCC after the commencement of NA therapy and underwent radical therapy for HCC. The recurrence rates of HCC were high in patients with high levels of intrahepatic cccDNA and serum HBcrAg. In particular, HBcrAg levels were measurable by using serum samples and clinically useful.

Nucleot(s)ide analogues, including LAM, ADV and ETV, are widely used for the treatment of chronic hepatitis B, and reportedly reduce the development of HCC in such patients (22, 23). Although few events of HCC development occur during NA therapy (24–26), analysis of a large number of patients is needed to examine the risk factors for HCC. We could clarify the risk factors associated with the development of primary HCC after radical therapy by enrolling patients who underwent radical therapy for HCC in spite of their small number. High HBV loads in serum have been reported to be associated with HCC recurrence after resection or radical therapy in NA-naïve patients (27–31), but no study has demonstrated the viral risk factors of recurrence in patients receiving NAs. The novel finding of this study is that serum HBcrAg and intrahepatic cccDNA levels are predictors of HCC recurrence in patients radically treated for HCC during NA therapy.

In this study, the serum HBV DNA levels at the time of HCC diagnosis were associated with recurrence by univariate analysis. However, the serum HBcrAg level was the only viral factor associated with recurrence in multivariate analysis. There are two possible reasons for the

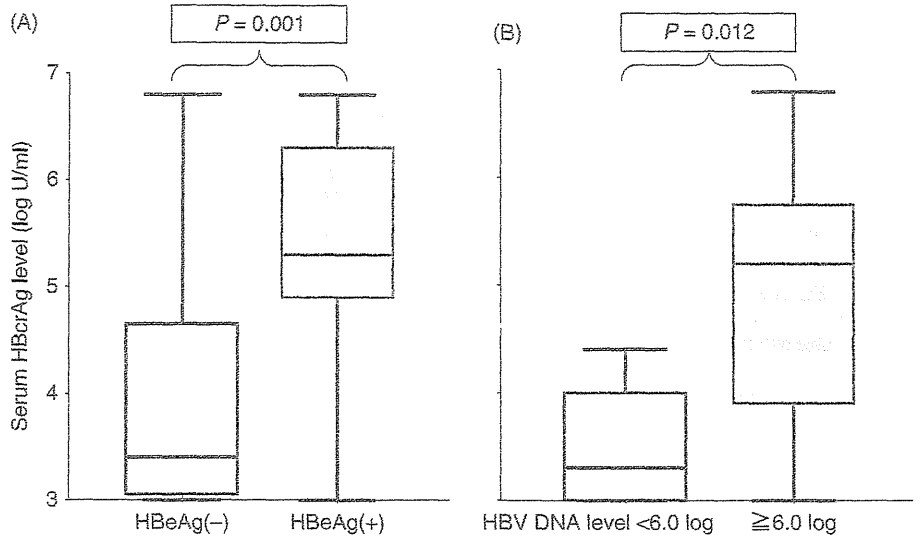

Fig. 5. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma diagnosis by the characteristics at the start of nucleot(s)ide analogue therapy (A) in patients with or without HBeAg and (B) in those with hepatitis B virus DNA (HBV DNA) levels < 6.0log or ≥6.0log copies/ml.

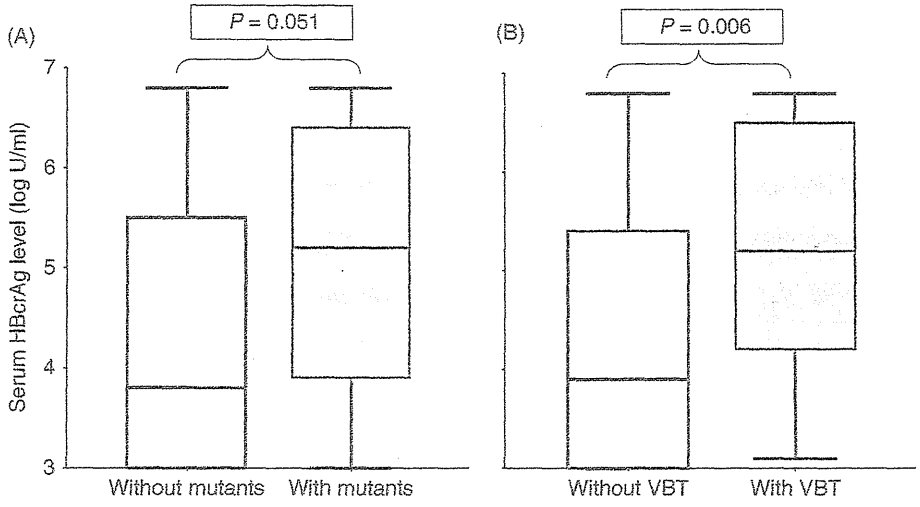

Fig. 6. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma (HCC) diagnosis (A) with or without thyrosine–methionine–aspartic acid–aspartic acid mutants and (B) virological breakthrough (VBT) before the development of HCC.

different results between past studies and the current study. Although serum HBV DNA was undetectable using TaqMan assay at the time of HCC diagnosis in 51% of the patients, who received NAs, serum HBcrAg was undetectable in only 18% of these patients. The other reason is that it was easy to identify the viral risk factors (e.g. HBeAg positivity) by measuring the serum HBcrAg level because the detection of HBcrAg enables the detection of HBcAg, HBeAg and the 22 kDa precore protein coded with the precore/core gene. The high recurrence rate of HCC after curative resection and ablation is attributable to two principal characteristics: intrahepatic metastasis and *de novo* multicentric carcinogenesis (32). It is assumed that a high viral load increases the risk of multicentric recurrence in the liver remnant in patients without optimal viral suppression by NA therapy. Recently, it was reported that the HBV load is associated with late recurrence over 2 years (30). On examining our cohort as per the recent report, high HBcrAg levels were found to be associated with late recurrence (data not shown). Consequently, we consider that HBcrAg is a more useful marker of HBV-related HCC recurrence than HBV DNA during NA therapy.

Nucleot(s)ide analogues are potent inhibitors of HBV replication, and can induce a rapid and drastic reduction in peripheral HBV DNA, seroclearance of HBeAg and remission of hepatic inflammation. Because of the stability of cccDNA in infected cells, the decline of

intrahepatic cccDNA levels is slower than that of serum HBV DNA levels during NA administration (15, 16). We found that suppression of cccDNA by NAs could prevent the development of recurrent primary HCC. Because cccDNA provides the template for pregenomic and viral messenger RNA-encoded viral proteins (33–35), the transcriptional activity of cccDNA may induce carcinogenesis. Further research is required to validate this hypothesis. Serum HBcrAg can be a surrogate marker of the intrahepatic cccDNA pool because of the viral proteins transcribed through messenger RNA from cccDNA (20, 21). Therefore, we consider that serum HBcrAg reflects the intrahepatic viral status more accurately than serum HBV DNA. Recently, Chan *et al.* (36) showed that serum HBsAg quantification could reflect intrahepatic cccDNA in patients treated with peginterferon and LAM combination therapy. They also indicated that reduction in HBsAg had good correlation with reduction in cccDNA. We tried to measure HBsAg levels at the start of NA therapy and the time of HCC diagnosis using a commercial assay (chemiluminescent immunoassay). However, HBsAg levels declined very slowly during NAs monotherapy in this study (data not shown). Brunetto *et al.* (37) showed that mean reduction for 48 weeks in HBsAg was 0.02log IU/ml in patients treated with LAM monotherapy, different from peginterferon therapy. Meanwhile, the median reduction from the start of NA to the diagnosis of HCC in HBcrAg was 1.4logU/ml in this study (Table 1). It seems that HBcrAg is a superior on-treatment risk predictor (e.g. tumour recurrence) to HBsAg during NAs monotherapy in terms of reduction of titres in each assay. HBcrAg is also more useful in terms of needless to serum sample dilution. As HBcrAg levels can be measured from serum samples, they are clinically useful, compared with the measurement of cccDNA, which requires liver specimens. It is not practical to carry out liver biopsy and the measurement of cccDNA for patients who have normal AST/ALT levels and viral suppression during antiviral therapy. Liver specimens cannot be also taken from patients who undergo ablation therapy for HCC. The measurement of serum HBcrAg levels in these patients is helpful to indirectly estimate the status of intrahepatic cccDNA. In the future, it is necessary to investigate whether HBcrAg in patients receiving NAs can be a predictor of primary carcinogenesis.

Previous studies have indicated that the rates of intrahepatic cccDNA loss and serum HBcrAg loss differ from serum HBV DNA loss under NA therapy, with the former two being much slower (15, 16, 19). In this study, the period of serum HBV DNA loss was longer, with lower intrahepatic cccDNA and serum HBcrAg levels (Fig. 2B). Therefore, these findings suggest that a long period of time is required to prevent the development of recurrent primary HCC by viral suppression under antiviral therapy. In contrast, the serum HBcrAg levels at the time of HCC diagnosis were higher in patients with emergent LAM-resistant mutants and subsequent VBT than in patients without mutants and VBT (Fig. 6). This result suggests that it is important to administer a potent NA early for drug-resistant strains and suppress viral replication to prevent subsequent carcinogenesis. Although we evaluated the relationship between the development of primary HCC and serum HBcrAg levels by a case–control study, the serum HBcrAg levels at the commencement of NA therapy and 1 year later were not associated with the development of primary HCC (unpublished data). This finding is attributable to the slow decline of the serum HBcrAg levels during antiviral therapy. The measurement of HBcrAg at intervals of 3–6 months may be helpful to predict the development of HCC. However, further studies are needed to confirm the finding.

In summary, HBcrAg is a predictor of the post-treatment recurrence of HCC during antiviral therapy. Measurement of the serum HBcrAg level is simple and useful because it reflects the intrahepatic viral status. Further, intrahepatic cccDNA and serum HBcrAg suppression by NAs is important to prevent HCC recurrence.

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References

1. Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; 337: 1733–45.
2. Ganem D, Prince AM. Hepatitis B virus infection – natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118–29.
3. Lai CL, Chien RN, Leung NW, *et al.* A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; 339: 61–8.
4. Dienstag JL, Schiff ER, Wright TL, *et al.* Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256–63.
5. Marcellin P, Chang TT, Lim SG, *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; 348: 808–16.
6. Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, *et al.* Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 2003; 348: 800–7.
7. Chang TT, Gish RG, de Man R, *et al.* A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001–10.
8. Lai CL, Shouval D, Lok AS, *et al.* Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; 354: 1011–20.

9. Lai CL, Gane E, Liaw YF, *et al.* Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; **357**: 2576–88.
10. Marcellin P, Heathcote EJ, Buti M, *et al.* Tenofovir disoproxil fumarate versus adefovir dipivoxil for chronic hepatitis B. *N Engl J Med* 2008; **359**: 2442–55.
11. Suzuki F, Suzuki Y, Tsubota A, *et al.* Mutations of polymerase, precore and core promoter gene in hepatitis B virus during 5-year lamivudine therapy. *J Hepatol* 2002; **37**: 824–30.
12. Akuta N, Suzuki F, Kobayashi M, *et al.* Virological and biochemical relapse according to YMDD motif mutant type during long-term lamivudine monotherapy. *J Med Virol* 2003; **71**: 504–10.
13. Lok AS, McMahon BJ. Chronic hepatitis B. *Hepatology* 2007; **45**: 507–39.
14. Yatsuji H, Suzuki F, Sezaki H, *et al.* Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. *J Hepatol* 2008; **48**: 923–31.
15. Werle-Lapostolle B, Bowden S, Locarnini S, *et al.* Persistence of cccDNA during the natural history of chronic hepatitis B and decline during adefovir dipivoxil therapy. *Gastroenterology* 2004; **126**: 1750–8.
16. Wursthorn K, Lutgehetmann M, Dandri M, *et al.* Peginterferon alpha-2b plus adefovir induce strong cccDNA decline and HBsAg reduction in patients with chronic hepatitis B. *Hepatology* 2006; **44**: 675–84.
17. Kimura T, Rokuhara A, Sakamoto Y, *et al.* Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. *J Clin Microbiol* 2002; **40**: 439–45.
18. Kimura T, Ohno N, Terada N, *et al.* Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore protein without C-terminal arginine-rich domain. *J Biol Chem* 2005; **280**: 21713–9.
19. Rokuhara A, Tanaka E, Matsumoto A, *et al.* Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen: a marker distinct from viral DNA for monitoring lamivudine treatment. *J Viral Hepat* 2003; **10**: 324–30.
20. Wong DK, Tanaka Y, Lai CL, *et al.* Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. *J Clin Microbiol* 2007; **45**: 3942–7.
21. Suzuki F, Miyakoshi H, Kobayashi M, Kumada H. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. *J Med Virol* 2009; **81**: 27–33.
22. Liaw YF, Sung JJ, Chow WC, *et al.* Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521–31.
23. Matsumoto A, Tanaka E, Rokuhara A, *et al.* Efficacy of lamivudine for preventing hepatocellular carcinoma in chronic hepatitis B: a multicenter retrospective study of 2795 patients. *Hepatol Res* 2005; **32**: 173–84.
24. Di Marco V, Marzano A, Lampertico P, *et al.* Clinical outcome of HBeAg-negative chronic hepatitis B in relation to virological response to lamivudine. *Hepatology* 2004; **40**: 883–91.
25. Lampertico P, Vigano M, Manenti E, *et al.* Low resistance to adefovir combined with lamivudine: a 3-year study of 145 lamivudine-resistant hepatitis B patients. *Gastroenterology* 2007; **133**: 1445–51.
26. Hosaka T, Suzuki F, Kobayashi M, *et al.* Development of HCC in patients receiving adefovir dipivoxil for lamivudine-resistant hepatitis B virus mutants. *Hepatol Res* 2010; **40**: 145–52.
27. Kubo S, Hirohashi K, Tanaka H, *et al.* Effect of viral status on recurrence after liver resection for patients with hepatitis B virus-related hepatocellular carcinoma. *Cancer* 2000; **88**: 1016–24.
28. Hung IF, Poon RT, Lai CL, *et al.* Recurrence of hepatitis B-related hepatocellular carcinoma is associated with high viral load at the time of resection. *Am J Gastroenterol* 2008; **103**: 1663–73.
29. Kim BK, Park JY, Kim do Y, *et al.* Persistent hepatitis B viral replication affects recurrence of hepatocellular carcinoma after curative resection. *Liver Int* 2008; **28**: 393–401.
30. Wu JC, Huang YH, Chau GY, *et al.* Risk factors for early and late recurrence in hepatitis B-related hepatocellular carcinoma. *J Hepatol* 2009; **51**: 890–7.
31. Jang JW, Choi JY, Bae SH, *et al.* The impact of hepatitis B viral load on recurrence after complete necrosis in patients with hepatocellular carcinoma who receive transarterial chemolipiodolization: implications for viral suppression to reduce the risk of cancer recurrence. *Cancer* 2007; **110**: 1760–7.
32. Llovet JM, Burroughs A, Bruix J. Hepatocellular carcinoma. *Lancet* 2003; **362**: 1907–17.
33. Wu TT, Coates L, Aldrich CE, Summers J, Mason WS. In hepatocytes infected with duck hepatitis B virus, the template for viral RNA synthesis is amplified by an intracellular pathway. *Virology* 1990; **175**: 255–61.
34. Newbold JE, Xin H, Tencza M, *et al.* The covalently closed duplex form of the hepadnavirus genome exists in situ as a heterogeneous population of viral minichromosomes. *J Virol* 1995; **69**: 3350–7.
35. Zoulim F. New insight on hepatitis B virus persistence from the study of intrahepatic viral cccDNA. *J Hepatol* 2005; **42**: 302–8.
36. Chan HL, Wong VW, Tse AM, *et al.* Serum hepatitis B surface antigen quantification can reflect hepatitis B virus in the liver and predict treatment response. *Clin Gastroenterol Hepatol* 2007; **5**: 1462–8.
37. Brunetto MR, Noriconi F, Bonino F, *et al.* Hepatitis B virus surface antigen levels: a guide to sustained response to peginterferon alfa-2a in HBeAg-negative chronic hepatitis B. *Hepatology* 2007; **49**: 1141–50.

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Original Article

New classification of dynamic computed tomography images predictive of malignant characteristics of hepatocellular carcinoma

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Aim: The aim of this study was to elucidate whether the histopathological characteristics of hepatocellular carcinoma (HCC) can be predicted from baseline dynamic computed tomography (CT) images.

Methods: This retrospective study included 86 consecutive patients with HCC who underwent surgical resection between January 2000 and September 2008. The arterial- and portal-phase dynamic CT images obtained preoperatively were classified into four enhancement patterns: Type-1 and Type-2 are homogeneous enhancement patterns without or with increased arterial blood flow, respectively; Type-3, heterogeneous enhancement pattern with septum-like structure; and Type-4, heterogeneous enhancement pattern with irregular ring-like structures. We also evaluated the predictive factors for poorly-differentiated HCC, specific macroscopic type of HCC (simple nodular type with extranodular growth [SNEG] and confluent multinodular [CMN]) by univariate and multivariate analyses.

Results: The percentages of poorly-differentiated HCC according to the enhancement pattern were three of 51 nodules (6%) of Type-1 and -2, three of 24 (13%) of Type-3, and eight of 11 (73%) of Type-4. The percentages of SNEG/CMN according to the enhancement pattern were 12 of 51 nodules (24%) of Type-1 and -2, 13 of 24 (54%) of Type-3, and five of 11 (45%) of Type-4. Multivariate analysis identified Type-4 pattern as a significant and independent predictor of poorly-differentiated HCC ($P < 0.001$) while Type-3 pattern was a significant predictor of SNEG/CMN ($P = 0.017$).

Conclusion: Heterogeneity of dynamic CT images correlates with malignant characteristics of HCC and can be potentially used to predict the malignant potential of HCC before treatment.

Key words: confluent multinodular type, dynamic computed tomography, hepatocellular carcinoma, poorly-differentiated hepatocellular carcinoma, radiofrequency ablation, simple nodular type with extranodular growth type.

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) is a common malignancy worldwide, and the incidence rate is increasing in Japan as well as in the USA.[1–3] Chronic viral hepatitis and liver cirrhosis following infection with hepatitis B virus (HBV) and hepatitis C virus (HCV) play important roles in the development of HCC.[4,5] The incidence of HCC in patients with HCV-related cirrhosis is estimated at 5–10% per annum, and it is one of the major causes of death, especially in Asian countries.[5] Among the available treatment options for HCC, surgical resection is generally considered a potentially curative method and could provide a satisfactory long-term outcome.[6–13] Recent advances in imaging procedures have led to increased detection of early-stage HCC and improved survival because of the greater number of patients identified in whom curative hepatic resection is possible.[14,15] However, for patients who are not suitable for surgical treatment for several reasons (e.g. lack of sufficient liver function for surgical resection), percutaneous local therapy is another therapeutic option. Various methods, such as percutaneous ethanol

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injection (PEI), percutaneous acetic acid injection (PAI), cryotherapy, percutaneous microwave coagulation therapy (PMCT) and radiofrequency ablation (RFA) are available for local therapy. In addition to surgical resection, local ablation therapy especially RFA is considered potentially curative for HCC and provides better long-term outcome.[16] However, despite the high complete necrosis rate in RFA, some patients show tumor recurrence within 1 year, either local recurrence or new tumor formation. A series of studies discussed the predictive factors involved in tumor recurrence and seeding including tumor size, subcapsular lesion, α-fetoprotein (AFP) levels, tumor staging and histopathological grading of HCC.[17,18] Another study has reported that the specific macroscopic type of HCC relevant to microvascular invasion on histopathological examination could help predict recurrence, and that this is especially true for simple nodular type with extranodular growth (SNEG) and confluent multinodular type (CMN) tumors.[19] For the above reasons, it is important to determine the histopathological grade and macroscopic type of HCC before the application of local ablation therapy.

One aim of the present study was to determine whether malignant characteristics of HCC (especially poorly-differentiated HCC, SNEG and CMN) can be diagnosed by dynamic computed tomography (CT) images obtained before treatment. We reported previously that angiographic hypervascularity corresponds with thick-walled, nuclei-rich, and slender-shaped non-triadal vessels (named "Type II vessels") identified by immunohistochemical staining for α-smooth muscle actin (α-SMA).[20] The other purpose of the present study was to correlate heterogenic enhancement pattern of the arterial- and portal-phase dynamic CT images with the distribution patterns of α-SMA-positive non-triadal vessels in HCC.

METHODS

Study population

FROM JANUARY 2000 to September 2008, 340 patients were diagnosed with HCC and received surgical resection as initial treatment in the Department of Hepatology, Toranomon Hospital, Tokyo, Japan. Among the 340 patients, 86 patients satisfied the following criteria: (i) triple-phase dynamic CT study was performed before surgical resection; (ii) preoperative diagnosis of a solitary HCC with a maximum tumor diameter of 50 mm; (iii) no evidence of extrahepatic metastases as confirmed by imaging studies (CT, ultrasonography [US] and chest X-ray) before procedure; (iv) no history of other malignancies; and (v) no preoperative chemotherapy including transcatheter chemoembolization. Accordingly, 86 patients with HCC who were underwent surgical resection for HCC were retrospectively evaluated for the relationship between heterogeneous enhancement pattern of the arterial- and portal-phase dynamic CT images and histopathological malignant characteristics of HCC. The observation starting point was the time of the first surgical resection for HCC.

Imaging analysis of HCC and definition of enhancement pattern

Before surgery, triple-phase contrast-enhanced CT was performed in all patients. In these studies, 95 mL of 350 mg I/mL Iomeprol (Iomeron 350, Eisai, Tokyo), as the contrast medium, was rapidly injected i.v. at 0.06 mL/kg bodyweight/s. Phase-1, -2, and -3 imaging were performed at 25, 60 and 180 s after the start of injection, respectively. The axial images were reconstructed at intervals of 5 mm. The enhancement pattern on the arterial- and portal-phase dynamic CT was classified into one of four types and the four enhancement types on the original images were converted into simplified images (Fig. 1). The Type-1 pattern represented a "homogeneous enhancement pattern with no increase in arterial blood flow"; the entire image was uniform during the arterial phase and portal phase. The Type-2 pattern represented "homogeneous enhancement pattern with increased arterial blood flow"; the entire image was uniform during the arterial phase and portal phase. The Type-3 pattern represented "heterogeneous enhancement pattern with septum-like structure"; with heterogeneous enhancement and septum-like formation in the arterial phase, while the septum-like structure resembled a near-uniform tumor tissue periphery in the portal phase. The Type-4 pattern represented "heterogeneous enhancement pattern with irregular ring-like structures" in the arterial phase; marked by the presence of irregularly-shaped ring areas of enhancement and areas of little blood flow relative to the periphery of the tumor tissue, and in the portal phase, by areas of reduced blood flow. The enhancement pattern on the arterial- and portal-phase dynamic CT was determined by three expert hepatologists blind to the pathological result.

Histopathological features

Tumor differentiation was graded histologically according to the classification of the Liver Cancer Study Group of Japan.[21] Macroscopic classification of nodular type