Table 2 Characteristics of the 18 patients at commencement of adefovir (ADV) and development of hepatocellular carcinoma (HCC)

Patient no.	Age (years)	Sex	At the commencement of ADV					YMDD mutant	Period of ADV (years)	At the development of HCC		
			Liver disease	AST (IU/L)	ALT (IU/L)	HBeAg	HBV DNA (log copies/mL)			AST (IU/L)	ALT (IU/L)	HBV DNA (log copies/mL)
1	50	M	CH	248	576	–	6.9	I	4.5	26	27	< 2.6
2	35	M	LC	217	164	+	7.5	I	1.6	54	34	< 2.6
3	50	M	LC	192	272	+	> 7.6	I	1.2	68	89	< 2.6
4	61	M	CH	192	332	–	6.9	I	2.8	22	23	< 2.6
5	65	M	CH	174	219	–	5.2	V	0.1	30	43	< 2.6
6	58	M	CH	160	216	–	6.5	V	2.2	41	32	< 2.6
7	53	M	LC	127	97	+	> 7.6	I	0.5	55	41	3.2
8	75	M	LC	119	209	+	> 7.6	V	1.1	121	125	2.6
9	58	F	CH	118	214	+	4.4	I	3.3	21	13	< 2.6
10	48	M	CH	116	99	+	> 7.6	I	3.3	32	36	< 2.6
11	51	F	LC	111	130	–	5.3	I	0.9	88	95	< 2.6
12	47	M	CH	85	138	+	> 7.6	I	1.3	28	29	3.1
13	61	M	LC	81	65	–	5.6	I	0.2	32	27	2.9
14	59	F	LC	80	132	–	> 7.6	V	0.1	32	41	3.2
15	40	M	LC	75	124	–	6.3	I	3.8	21	24	< 2.6
16	48	M	CH	71	61	–	6.6	I	0.6	48	26	3.7
17	55	M	LC	55	76	+	7.3	I	0.2	50	64	5.4
18	43	M	LC	27	21	–	5.4	V	1.6	30	23	3.7

ALT, alaine aminotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; I, YIDD mutant; LC, cirrhosis; V, YVDD mutant.

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Table 3 Independent risk factors influencing the development of hepatocellular carcinoma

Factors	Category	Hazard ratio (95% CI†)	*P*-value
AST (IU/l)	1: < 70	1	0.016
	2: ≥ 70	6.21 (1.40–27.5)	
YMDD mutants	1: YVDD or YV/IDD	1	0.012
	2: YIDD	3.97 (1.36–11.6)	
Age (years)	1: < 50	1	0.023
	2: ≥ 50	3.24 (1.17–8.95)	
Cirrhosis	1: Absent	1	0.030
	2: Present	1.42 (1.04–1.96)	

†Confidence interval.

adefovir was 59 (0–896) days for the patients who developed HCC and 54 (0–3240) days for those who did not ($P = 0.330$). Hence, exacerbation of hepatitis was not a risk factor for the development of HCC.

Age-specific risk factors for the development of HCC were evaluated by the multivariate analysis. In the patients < 50 years, platelet counts < $13 \times 10^3/mm^3$ was the only significant risk factor for HCC (hazard ratio 6.88 [95% confidence interval; 1.26–37.6]), while AST levels ≥ 70 IU/L was that in those ≥ 50 years (hazard ratio: 9.50 [95% confidence interval 1.20–74.9]).

Factors increasing the cumulative incidence of hepatocellular carcinoma

AST levels ≥ 70 IU/L at the start of adefovir increased the development of HCC during follow-ups ranging to 5 years (Fig. 1). HCC developed more frequently in the patients with YIDD mutants than in those with YVDD or the mixture of YVDD and YIDD mutants (Fig. 2). The cumulative incidence of HCC in the patients with YIDD mutants alone was: 4% at 1 year, 10% at 3 years and 43% at 5 years. In contrast, HCC never developed in the patients with the mixture of YIDD and YVDD mutants through 5 years of follow-up. HCC developed more frequently in the patients with cirrhosis and those aged ≥ 50 years (Figs 3,4, respectively).

DISCUSSION

HCC DEVELOPED IN 18 of the 247 (7.3%) patients who had received adefovir add-on lamivudine during a long-term ranging to 5 years. There were some differences in the characteristics at the start of adefovir dipivoxil between the patients who did and who did not develop HCC. The patients who developed HCC were older, more frequently had signs of early cirrhosis with less platelet counts, as well as higher levels of AST, ALT and AFP, than those who did not develop HCC. By multivariate analysis, AST ≥ 70 IU/L, YIDD mutants in

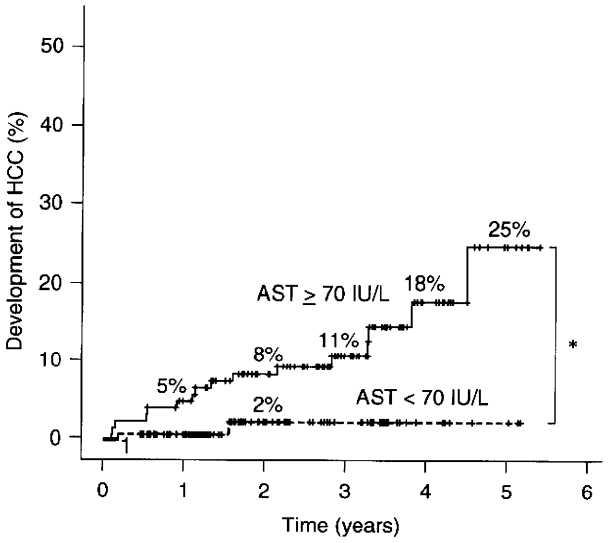

Figure 1 Kaplan–Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients with different baseline aspartate aminotransferase (AST) levels. *$P = 0.009$.

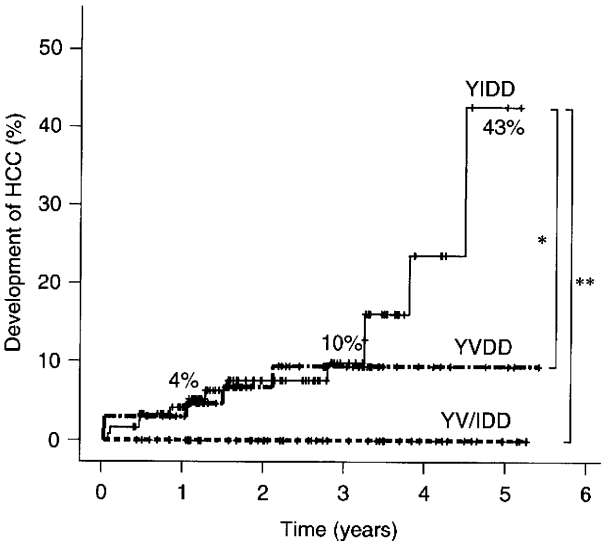

Figure 2 Kaplan–Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adeforvir add-on lamivudine in the patients with distinct YMDD mutants. *$P = 0.035$; **$P = 0.003$.

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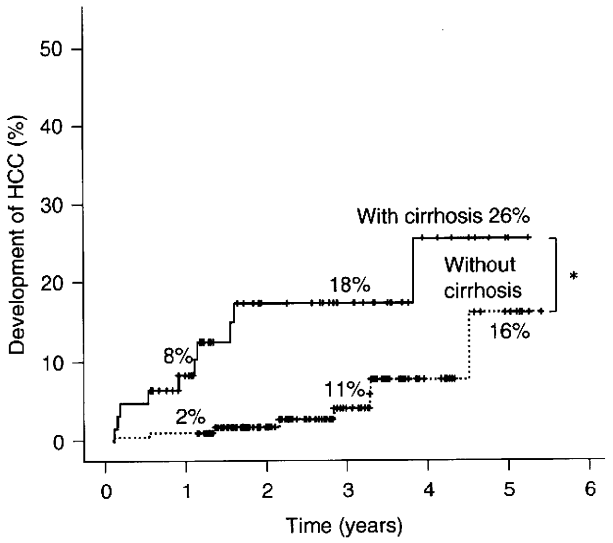

Figure 3 Kaplan–Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients with and without cirrhosis at the baseline. $^*P = 0.002$.

comparison with YVDD or the mixture of YVDD and YIDD mutants, age ≥ 50 years and cirrhosis were independent risk factors for the development of HCC. By the Kaplan-Meier life-table analysis, the cumulative incidence of HCC during 5 years in the patients receiving

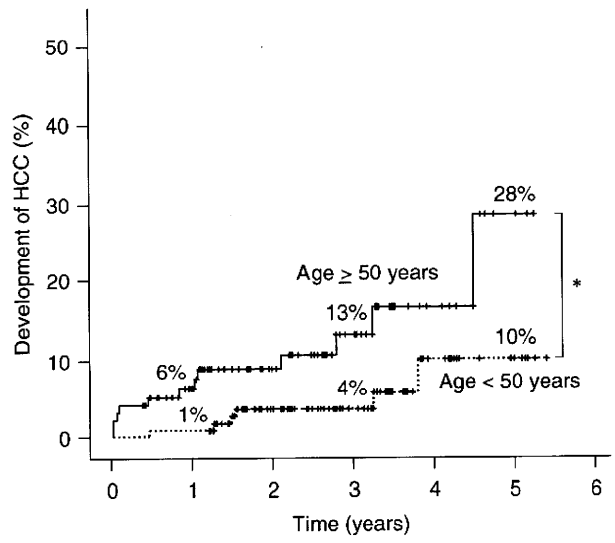

Figure 4 Kaplan–Meier life-table for the cumulative incidence of hepatocellular carcinoma (HCC) during adefovir add-on lamivudine in the patients aged ≥ 50 years and < 50 yeas at the baseline. $^*P = 0.014$.

adefovir add-on lamivudine was significantly higher in those with AST ≥ 70 IU/L, YIDD mutants, cirrhosis and aged ≥ 50 years at the start of adefovir.

A marked difference in the development of HCC between the present study (7.3% [18/247]) and two studies reported from Europe and the US (0/70 and 0/65, respectively)[16,17] would be accounted for, at least in part, by the age of patients who developed HCC in this study that was older than in those in previous reports (the median of 52 years vs. means of 36 and 47 years, respectively). This view would be supported by the age of patients with long-term adefovir add-on lamivudine that was higher in those with than without the development of HCC (52 vs. 45 years [median], $P = 0.008$). HBV infection in Asia is acquired by the perinatal infection, while that in Western countries is gained after the adolescence ~20 years after birth. Hence, the duration of HBV infection would have been > 20 years longer in Japanese than Western patients. In addition, genotypes of HBV may give an additional account on the difference in development of HCC between them. All the 18 patients who developed HCC in this study were infected with genotype C; it is associated with HCC more closely than the other genotypes.[20-23] By contrast, by far the most patients from Western countries would have been infected with genotypes A and D.[24,25]

HCC developed more frequently in patients with than without cirrhosis at the start of adefovir (10/61 [16.4%] vs. 8/186 [4.3%], $P = 0.002$). Hence, cirrhosis increased the risk of HCC in patients receiving adefovir add-on lamivudine. This view is supported by the development of HCC in 11 of the 94 (11.7%) patients with cirrhosis who received adefovir add-on lamivudine from Italy.[10] Although HCC did not develop in any of the 39 Italian patients with chronic hepatitis, it did in eight of the 186 (4.3%) Japanese patients in the present study. There were, however, marked differences in the median baseline ALT levels between Italian and Japanese patients (58 vs. 108 IU/L); the grade of liver inflammation would have been higher in the Japanese patients. In actuality, all the eight patients with chronic hepatitis who developed HCC had high AST and ALT levels at the start of adefovir (Table 2).

In the natural history of persistent HBV infection, HCC develops more frequently in the patients with persistently high ALT levels than in those with normal levels. Hence, necroinflammation in the liver would contribute to carcinogenesis.[26,27] Although adefovir add-on lamivudine may prevent virological breakthroughs, it would not be able to suppress the pre-

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neoplastic state induced by exacerbation of hepatitis. It would be necessary therefore to identify the patients with chronic hepatitis at an increased risk for HCC during adefovir add-on lamivudine, such as those with cirrhosis or aged ≥ 50 years, and take special care of them toward early detection of HCC and immediate therapeutic intervention. They need to be monitored frequently for any increase in HBV DNA and aminotransferase levels that herald breakthrough hepatitis during lamivudine therapy.

In the present study, HCC developed more frequently in the patients with YIDD mutants than in those with YVDD or the mixture of YVDD and YIDD; there have been no studies correlating YMDD mutants and the development of HCC. No patients with the mixture of YVDD and YIDD mutants developed HCC, despite the predominance of YIDD mutants in the patients with HCC. This might have been due to the assay used for YMDD mutants by the commercial kit; it can miss YVDD mutants in samples in which YIDD mutants account for the great majority. By the assay method specific for either mutant, YIDD was detected either alone or accompanied by small amount of YVDD in the patients who have received adefovir add-on lamivudine treatment.[28] Sensitive and specific quantification of YIDD and YVDD mutants are necessary for further evaluating a role for YIDD mutants in hepatocarcinogenesis, as well as for identifying factors promoting the generation of both YIDD mutants and HCC.

Some points of clinical importance have emerged in the present study. First, patients who receive a long-term adefovir add-on lamivudine and have developed YMDD mutants need to be screened for HCC on the regular basis. This is required especially for the patients who have signs of cirrhosis and/or high AST levels, or aged ≥ 50 years. In these high-risk patients, adefovir has to be started promptly when HBV DNA levels increase, even before transaminase levels elevate in them. Secondly, it would be a matter of concern if adefovir is involved in the development of HCC. Should it be the case, tenofovir or newer potent antivirals, either as a monotherapy or add-on lamivudine, would deserve considerations. Thirdly, it needs to be evaluated if YIDD mutants have any significance in the development of HCC. Although nucleot(s)ide analogues may suppress hepatic inflammation and are expected to improve the prognosis of patients with chronic hepatitis B, they need to be monitored closely for HCC. The development of HCC has to be identified, as early as possible, for timely treatment toward longevity with minimal morbidity and improvement of the quality of life.

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CLINICAL STUDIES

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

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Keywords
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

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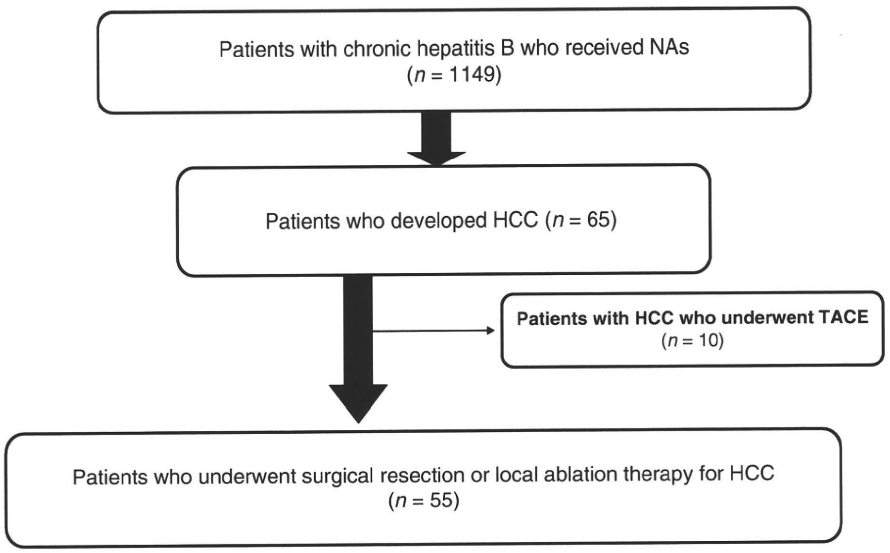

Fig. 1. The study protocol. HCC, hepatocellular carcinoma; NAs, nucleot(s)ide analogues; TACE, transcatheter arterial chemoembolization.

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

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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 ≥ 1log 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

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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 ($P < 0.001$; $r = -0.568$).

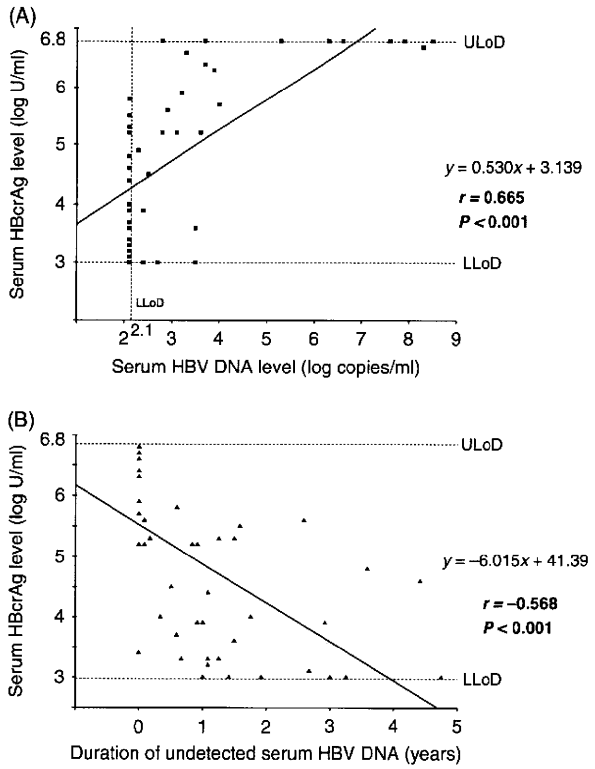

Fig. 2. (A) Correlation between serum HBcrAg and hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient. (B) Correlation between serum HBcrAg levels at the time of HCC diagnosis and the duration of undetected serum HBV DNA (< 2.6log copies/ml).

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

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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

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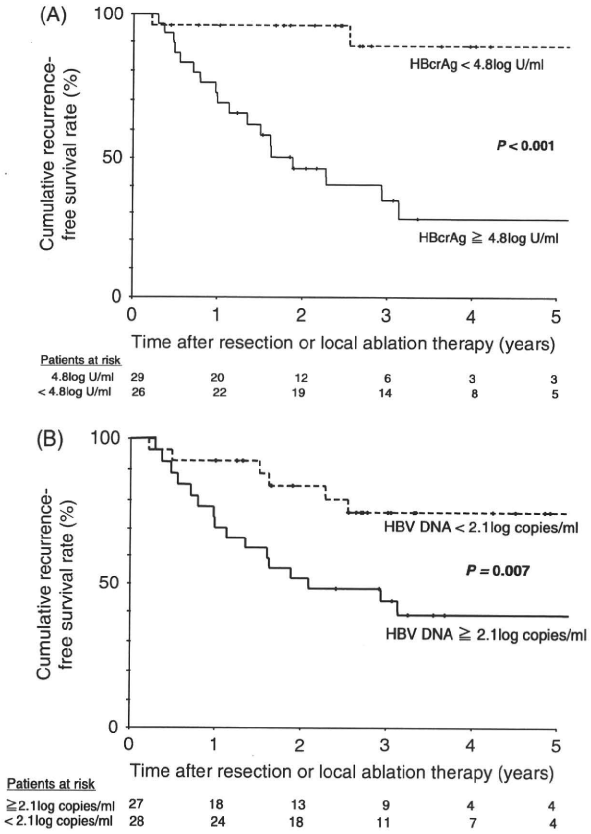

Fig. 3. (A) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum HBcrAg levels and comparison by the log-rank test. (B) Kaplan–Meier life table for the cumulative recurrence-free survival rates by the serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular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

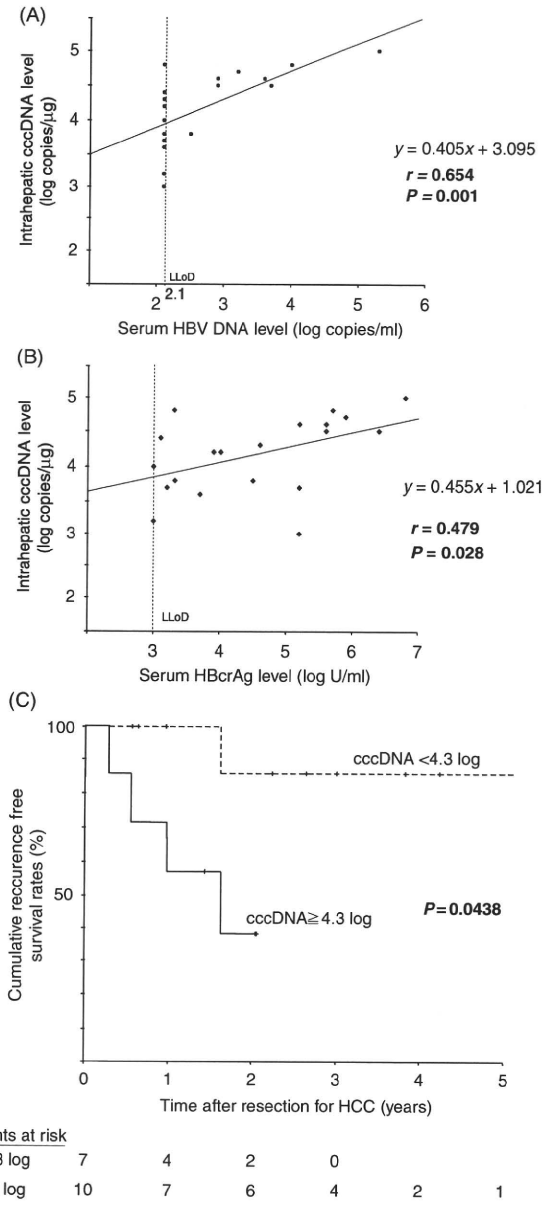

Fig. 4. (A) Correlation between intrahepatic covalently closed circular DNA (cccDNA) and serum hepatitis B virus DNA (HBV DNA) levels at the time of hepatocellular carcinoma (HCC) diagnosis for each patient who underwent resection ($n = 22$). (B) Correlation between intrahepatic cccDNA and serum HBcrAg 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

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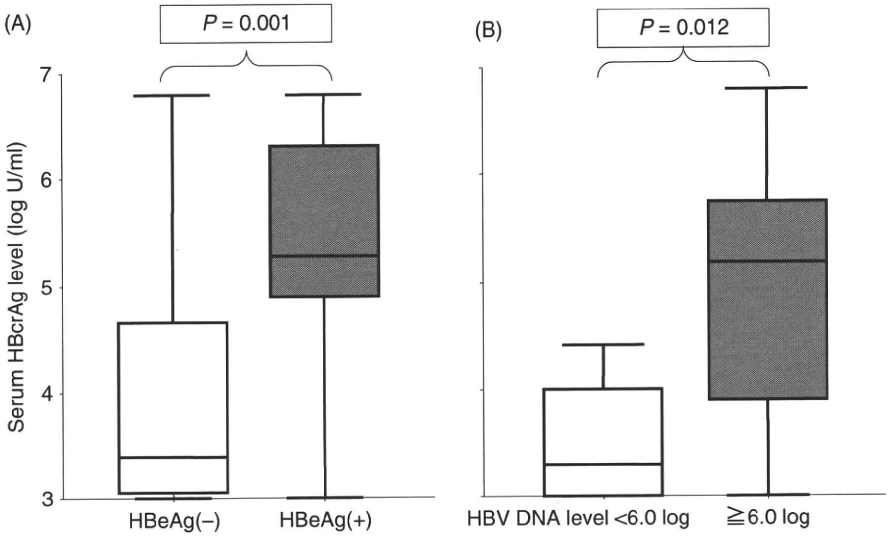

Fig. 5. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma diagnosis by the characteristics at the start of nucleot(s)ide analogue therapy (A) in patients with or without HBeAg and (B) in those with hepatitis B virus DNA (HBV DNA) levels < 6.0log or ≥6.0log copies/ml.

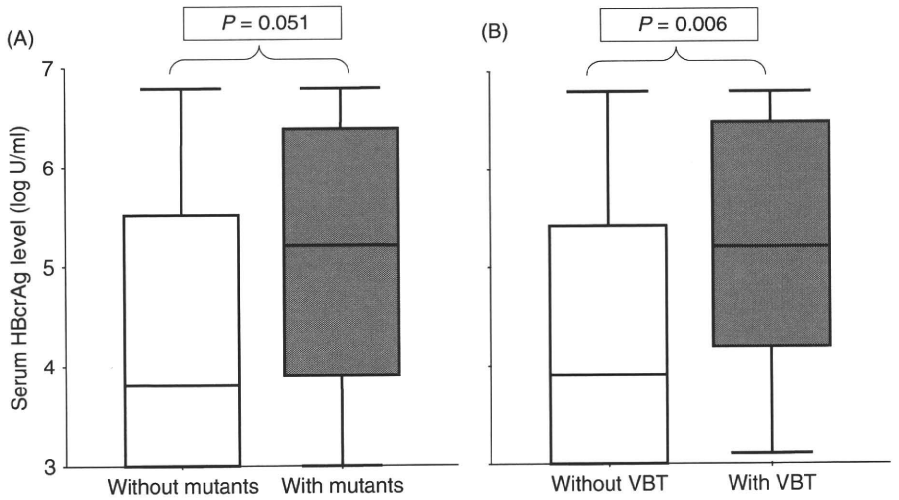

Fig. 6. Comparison of serum HBcrAg levels at the time of hepatocellular carcinoma (HCC) diagnosis (A) with or without thyrosine–methionine–aspartic acid–aspartic acid mutants and (B) virological breakthrough (VBT) before the development of HCC.

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

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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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Original Article

Administration of interferon for two or more years decreases early stage hepatocellular carcinoma recurrence rate after radical ablation: A retrospective study of hepatitis C virus-related liver cancer

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Background: Since hepatocellular carcinoma often recurs after surgical resection or radiofrequency ablation, we analyzed a retrospective large cohort of patients with small hepatocellular carcinoma caused by hepatitis C virus (HCV).

Methods: Among 379 patients with HCV RNA-positive small hepatocellular carcinoma (multiple up to three nodules, 3 cm or less each), 77 received interferon-alpha injection and 302 received no anti-viral therapy.

Results: Four patients (5.2%) attained sustained virological response (SVR). Cumulative recurrence rates in the treated and untreated groups were 41.1% and 57.5% at the end of the third year, and 63.0% and 74.5% at the fifth year, respectively ($P = 0.013$). Fifth year-recurrence rates in treated group were 25.0% in SVR, 85.7% in biochemical response, 71.1% in no response, and 46.7% in patients with continuous administration. When four patients with SVR were excluded, recurrence rates in short-term interferon therapy (<2 years) and long-term therapy (≥2 years) were 46.2% and 39.3% at the third year, and 66.2% and 57.4% at the fifth year, respectively ($P = 0.012$). Multivariate analysis showed that long-term interferon therapy significantly decreased recurrence rate (hazard ratio for interferon <2 years 0.80, interferon ≥2 years 0.60, $P = 0.044$), after adjustment with background covariates including indocyanine green retention rate ($P = 0.018$), alpha-fetoprotein ($P = 0.051$), and tumor treatment ($P = 0.066$).

Conclusion: A long-term administration of low-dose interferon significantly decreased recurrence of hepatocellular carcinoma after surgical resection or radiofrequency ablation.

Key words: hepatitis C, hepatocellular carcinoma, Interferon, prevention, recurrence

INTRODUCTION

HEPATOCELLULAR CARCINOMA (HCC) remains one of the most common cancers, and cause of cancer death, worldwide. Since the recurrence rate of HCC is high even after potentially curative therapies with surgical resection or radiofrequency ablation (RFA) therapy, suppression of recurrence is of great importance for prolonging the life of patients with hepatitis C virus (HCV)-related liver disease. This high recurrence rate, after curative therapy, was explained by occult intra-hepatic metastasis of HCC or by multi-centric carcinogenesis in the setting of chronic viral hepatitis or liver cirrhosis.[1,2]

Interferon (IFN) is effective in reducing hepatocellular carcinogenesis rate through suppression of necro-inflammatory process and in eliminating HCV in some patients with chronic hepatitis C and cirrhosis. Although IFN proves to be valuable in suppression of the risk of carcinogenesis in many literatures,[3–5] only several reports mentioned the efficacy of IFN in the suppression of tumor recurrence or in prolongation of survival period after ablation of HCC[6–12]. We once

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demonstrated the preventive activity of HCC recurrence by IFN-beta in a randomized controlled trial,[5] but intravenous type of IFN-beta was not universally available outside Japan in spite of the superiority of tumor suppressive activity to IFN-alpha.[13–17] Some investigators[14–17] showed that IFN acted as an anti-cancer agent in the treatment of HCC *in vivo* and *in vitro*. However, the actual efficacy of IFN in preventing recurrence of HCV-associated HCC in optimally treated patients remains unclear. Since some prospective study failed to demonstrate a beneficial effect of IFN-alpha in cumulative recurrence rate,[11] we analyzed a large cohort of patients for a long period up to 18 years.

To what extent IFN suppresses the recurrence rate of early stage of HCC, we analyzed a large retrospective cohort with and without a long-term administration of IFN-alpha in patients with HCC. The purposes of this study were (i) to evaluate the influence of IFN-alpha on HCC recurrence rate after treatment of an early stage of HCV-related HCC, and (ii) to explore effective ways of IFN administration, if any.

PATIENTS AND METHODS

Study population

A TOTAL OF 729 patients were diagnosed as having HCC associated with HCV-related chronic liver disease from 1990 to 2006 in our hospital. Among them, 379 patients underwent surgical resection or sufficient medical ablation therapy for small HCC (multiple up to three nodules, 3 cm or less each). All were positive for anti-hepatitis C antibody and negative for hepatitis B surface antigen. The consecutive patients were analyzed, who met inclusion criteria of (1) initial diagnosis of HCC (2) early stage of HCC (multiple up to three nodules, 3 cm or less each) (3) potentially curative manner of resection or radiofrequency ablation for HCC, and (4) positive HCV RNA. Exclusion criteria of this study were (1) positive portal vein invasion on imaging of computerized tomography or ultrasonography (2) residual HCC on imaging diagnosis after surgical or medical therapy (3) Child-Pugh score C (4) other etiology of liver disease (hepatitis B, alcoholic, non-alcoholic liver disease, etc.) (5) use of other anti-viral agents including interferon-beta (6) use of retinoid derivatives, and (7) concomitant malignant tumor in addition to HCC.

The diagnosis of HCC was established by integrated imagings of ultrasonography, dynamic computerized tomography (CT), magnetic resonance imaging (MRI). To exclude additional small HCC nodules in the liver, computerized tomographic hepatic arteriography (CT-HA) and computerized tomographic arterioportography (CT-AP) were also performed in 356 patients (93.9%). Among the consecutive 379 patients with surgical resection or sufficient radiofrequency ablation for HCC, 77 (20.3%) patients received intermittent IFN-alpha injection two or three times a week for 6 months or longer, mainly after the year of 1995 when this medication became available for use in Japan: Two (3.4%) of 59 patients received IFN therapy during 1990–1994, 21 (21.2%) of 99 patients during 1995–2000, and 54 (24.2%) of 223 patients during 2001–2006, respectively. The other 302 patients did not receive IFN therapy or other anti-viral therapy. None of the patients received any other anti-viral or anti-carcinogenic treatment including nucleoside analogues. We therefore, performed this analytical study as a retrospective cohort study.

Clinical background and laboratory data

Table 1 summarizes the profiles and laboratory data of the IFN group (group A) and the untreated group (group B) at the time of diagnosis of HCC. The median age in the IFN group was lower than that of the untreated group by 3 years, but the other features were not different between the two groups regarding demography, liver function, state of HCC, and treatment of HCC.

Interferon treatment and judgment of the effect

Seventy-seven patients underwent IFN therapy after treatment of HCC. IFN therapy was usually initiated within several months after ablation of HCC, and a median period from HCC treatment to initiation of IFN was 5.6 months.

All the patients received IFN-alpha (natural or recombinant): Seven received interferon plus ribavirin combination therapy, and 68 underwent interferon monotherapy. Ten patients (13.0%) underwent interferon therapy for 6 months or less, 15 patients (19.5%) for 7 to 12 months, 13 patients (16.8%) for 13 to 24 months, 28 (36.4%) for 25 to 60 months, and the remaining 11 (14.3%) for a prolonged period of 61 months or longer. As a whole, a median dose of 242 million units was administered during the median period of 24.2 months. A total of 50.6% of all the patients received IFN for 2 years or longer.

Judgment of IFN effect was classified according to elimination of HCV RNA and alanine aminotransferase (ALT) value at a time of 6 months after the end of the

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Table 1 Profiles and laboratory tests of the patients with and without interferon

Groups/characteristic	Group A (interferon)	Group B (none)	*P**
Patients characteristics			
N	77	302	
Age (year) (median, range)	63 (43–77)	66 (39–87)	0.003
Sex (Male/Female)	46/31	191/111	0.57
Positive HBs antigen	0	0	NS
Positive HCV antibody	77 (100%)	302 (100%)	NS
Positive HCV-RNA	77 (100%)	302 (100%)	NS
Cancer characteristics before treatment			
Number of nodules			0.89
Solitary	63	260	
Two	11	33	
Three	3	9	
Size of maximal tumor (median, range)	18 (5–30)	18 (8–30)	0.50
Vascular invasion on imaging	0	0	NS
Cancer therapy			
Surgery	35 (45.5%)	146 (48.3%)	0.65
Radiofrequency ablation	42 (54.5%)	156 (51.7%)	
Laboraory findings (median, range)			
Albumin (g/dl)	3.6 (2.4–4.3)	3.6 (2.4–4.5)	0.80
Bilirubin (mg/dl)	1.0 (0.3–2.5)	1.0 (0.2–3.3)	0.96
Aspartic transaminase (IU)	54 (16–311)	54.5 (13–191)	0.94
Alanine transaminase (IU)	57 (12–273)	54 (11–230)	0.89
Platelet (×1000/cmm)	100 (20–272)	110 (20–256)	0.85
ICG R15 (%)	25 (1–75)	27 (2–78)	0.58
Alpha-fetoprotein (mg/L)	22 (3–1411)	22 (1–4950)	0.28
DCP (AU/L)	19 (11–635)	17 (0–1470)	0.50

*Non-parametric test (χ^2 test or Mann–Whitney *U*-test). DCP, des-gamma-carboxyprothrombin; ICG R15, indocyanine green retention test at 15 minutes.

treatment. Sustained virological response (SVR) was defined as persistent disappearance of HCV RNA after therapy, biochemical response (BR) as normal ALT values (40 IU/L or less) without elimination of HCV RNA for at least 6 months after therapy, and no response (NR) as persistently abnormal or only transient normalization of ALT for less than 6 months.

Follow-up and diagnosis of HCC

Physicians examined the patients every 4 weeks after entry to the study. Liver function tests and hematologic and virologic tests were conducted every month. To diagnose recurrent HCC nodules at an early stage, imaging studies were performed every 3 months, using ultrasonography and computerized tomography. Alfa-fetoprotein and des-gamma-carboxyprothrombin were also assayed bimonthly. When angiography demonstrated a characteristic hypervascular nodule, it was usually a specific finding for HCC in these follow-up patients, and histological confirmation was usually not required in the majority of these HCC patients. Most of the "angiographically-diagnosed HCC" showed intrahepatic multiplicity and pathognomonic findings of capsule formation or nodule-in-nodule appearance, or even portal vein invasion. If angiography did not show any hypervascular stain in a small hepatic nodule, histological study was always performed.

A total of 8 patients could not continue the IFN treatment due to side effects, following studies of tumor recurrence and survival were analyzed on an intention-to-treat basis.

Eight patients were lost to follow-up: 2 in IFN group and 6 in untreated group. Treated and untreated patients were followed at intervals of one month for a median observation period of 4.6 years, ranged from 0.1 to 18.4 years: 5.6 years in interferon group and 4.2 years in untreated group. The date of the last follow-up for this study was 30th August, 2009.

The end point of the study was tumor recurrence after treatment.

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Statistical methods

The obtained clinical data were analyzed on an intention-to-treat basis. Standard statistical measures and procedures were used in the analysis. The chi-square, Fisher's exact test, and Mann-Whitney's *U*-tests were used to analyze the differences of background features and biochemical data between the two groups. HCC recurrence rate was calculated from the day of HCC treatment in both groups, using the Kaplan-Meier technique. The differences in recurrence curves were tested using the log-rank test. Cox proportional hazard analysis was performed to evaluate independent predictors of tumor recurrence after treatment. A *P*-value of less than 0.05 with two-tailed analysis was considered significant. Data analysis was performed using the computer program SPSS version 11 (SPSS Inc. Chicago, IL).[18]

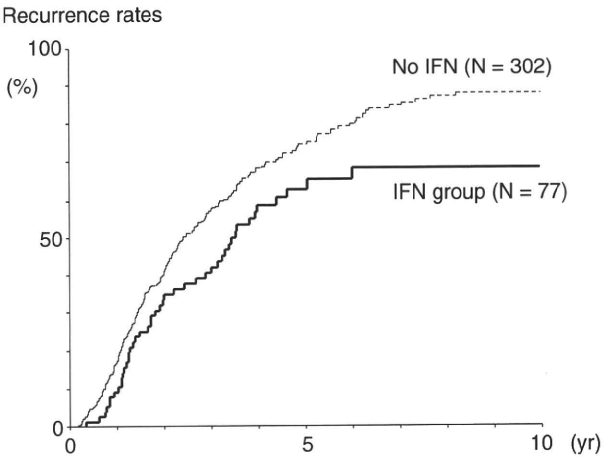

Figure 1 Cumulative recurrence rates of hepatocellular carcinoma in patients with and without interferon therapy.

RESULTS

Effects and toxicity of interferon

SVR WERE FOUND in 4 (5.2%) of 77 patients in IFN-treated group and none in untreated group. BR were found in 7 (9.1%), NR in 36 (46.8%), and undetermined judgment due to continuous administration currently in 30 (39.0%).

Almost all of the patients given IFN therapy showed varied degrees of fever, chills, myalgias, headache, and general malaise after the first injection of IFN. Most of patients revealed a various degree of leukocytopenia and thrombocytopenia. A total of 8 patients (10.4%) withdrew from IFN therapy before development of tumor recurrence. Three patients with depression or psychosis ceased the IFN therapy. The other 5 patients also stopped IFN administration because of varied degree of adverse effects: thrombocytopenia, insomnia, slight degree of hepatic encephalopathy, minor episode of cerebrovascular accident, and generalized fatigue with significant weight loss.

Recurrence rates of hepatocellular carcinoma

During the median observation period of 4.6 years, HCC recurred in 264 patients (69.7%); 45 patients belonged to the IFN group, and the other 219 patients to the untreated group. The cumulative recurrence rate in all patients was 16.2% at the end of the first year following the surgical treatment of HCC, 39.6% at the second year, 54.5% at the third year, 73.0% at the fifth year, 82.8% at the seventh year, and 85.5% at the 10th year. Crude recurrence rates in the IFN group and untreated group were 9.1% and 18.8% at the end of the first year, 33.3% and 42.1% at the second year, 41.1% and 58.1% at the third year, 63.0% and 76.6% at the 5th year, 68.5% and 86.2% at the seventh year, and 68.5% and 93.2% at the 10th year, respectively (Fig. 1). The recurrence rate in the IFN group was significantly lower than that of the untreated group (log-rank test: $P = 0.013$).

In univariate analysis, factors associated with tumor recurrence were explored in all of the 379 patients *en masse*. HCC recurrence was associated with high indocyanine green retention rate at 15 minutes (ICG R15) ($P = 0.004$), low albumin concentration ($P = 0.005$), no IFN therapy ($P = 0.010$), prolonged prothrombin time ($P = 0.041$), and RFA as treatment for HCC ($P = 0.046$).

Multivariate analysis disclosed that recurrence of HCC was independently associated with IFN therapy (hazard ratio 0.66, $P = 0.020$), a high ICG R15 of 20% or more (hazard ratio 1.43, $P = 0.008$), and RFA therapy (hazard ratio 1.32, $P = 0.041$). IFN treatment proved to prevent tumor recurrence after ablation of HCC in those patients with an early stage of HCC (Table 2).

Recurrence rates according to interferon effect

Tumor recurrence rates were evaluated according to judgment of IFN effect in the treated group: SVR ($n = 4$), BR ($n = 7$), NR ($n = 36$), continued IFN administration (N = 30), and untreated group.

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Table 2 Independent factors affecting the recurrence of hepatocellular carcinoma after curative treatment

Factors	Category	Hazard ratio (95% CI)	*P*
Interferon therapy	1: No	1	
	2: Yes	0.66 (0.46–0.94)	0.020
ICG R15	1: <20%	1	
	2: ≥20%	1.43 (1.10–1.85)	0.008
Cancer treatment	1: Surgical resection	1	
	2: PRFA	1.32 (1.01–1.72)	0.041

ICG R15, indocyanine green retention rate at 15 minutes; PRFA, percutaneous radiofrequency ablation therapy.

Recurrence rates in the subgroup of SVR, BR, NR, continued administration, and untreated patients were 0%, 0%, 6.7%, 12.5%, and 18.8% at the end of the first year, 25.0%, 28.6%, 37.0%, 25.3%, and 42.1% at the second year, 25.0%, 42.9%, 52.0%, 32.6%, and 58.1% at the third year, 25.0%, 85.7%, 71.1%, 46.7%, and 76.6% at the fifth year, and 25.0%, 100%, 79.3%, 54.3%, and 86.2% at the seventh year, respectively (Fig. 2a). The recurrence rates in a combined group of SVR and continued IFN administration were significantly lower than those in a combined cohort of the other groups (log-rank test, $P = 0.0005$) (Fig. 2b). The recurrence rates of the former and the latter groups were 30.6% and 56.7% at the end of the third year, 43.3% and 75.0% at the fifth year, and 43.3% and 84.7% at the seventh year, respectively.

Recurrence rates according to length of interferon administration

Since HCV RNA eradication (SVR) was found in only four patients, significance of prolonged administration of IFN was assessed in those patients with positive HCV RNA during therapy ($n = 73$).

Recurrence rates in the subgroup with a long IFN therapy of 2 years or more ($n = 39$), a short IFN therapy of less than 2 years ($n = 34$), and in the untreated patients ($n = 302$) were 8.7%, 7.1%, and 18.8% at the end of the first year, 23.9%, 40.2%, and 42.1% at the second year, 39.3%, 46.2%, and 58.1% at the third year, 57.4%, 66.2%, 76.6% at the fifth year, and 66.0%, 77.5%, and 86.2% at the seventh year, and 66.0%, 77.5%, and 93.2%, respectively (Fig. 3). The recurrence rates in the long IFN-therapy group was significantly lower than those with a short therapy group and untreated group (log-rank test, $P = 0.012$).

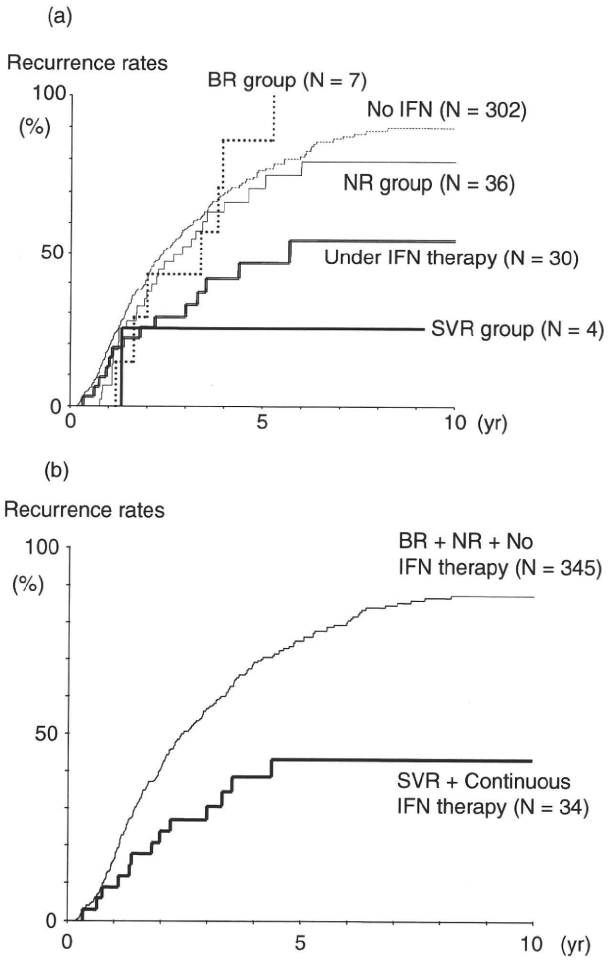

Figure 2 (a) Cumulative recurrence rates of hepatocellular carcinoma according to the effect of interferon. (b) Cumulative recurrence rates of hepatocellular carcinoma in a combined group of sustained virological response and continuous interferon administration and those in a combined group of biochemical response, no response, and no interferon therapy.

To elucidate the impact of a long-term administration of IFN in the prevention of HCC recurrence, multivariate hazard analysis was introduced in the IFN-treated patients without SVR effect ($n = 73$) and the untreated patients ($n = 302$). Multivariate analysis showed that a long-term IFN therapy significantly lowered the recurrence rate in patients with HCV-related HCC: hazard ratios of short-term therapy less than two years and long-term therapy for two years or longer of 2 years or more were 0.80 and 0.60, respectively ($P = 0.044$). The other covariates for recurrence rate included high ICGR15, high AFP value, and initial treatment modality (Table 3).

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