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Correlation Between Serum Hepatitis B Virus Core-Related Antigen and Intrahepatic Covalently Closed Circular DNA in Chronic Hepatitis B Patients

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Nucleos(t)ide analogues are utilized for the treatment of chronic HBV infection, and HBe seroconversion and HBV DNA levels are commonly used as markers of viral status and as primary treatment endpoints. Recently, a new assay was prepared for the detection of serum HBV core-related antigen (HBcrAg), consisting of HBcAg, HBeAg, and p22cr, which is a precore protein from amino acid −28 to at least amino acid 150, by coding the precore/core region. In this study, we examined the correlation between serum HBcrAg concentration and viral status by the analysis of serum HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic covalently closed circular DNA (cccDNA) in 57 chronic hepatitis B patients. Intrahepatic cccDNA was detected in all 57 patients, 42 patients were HBcrAg-positive, and serum HBcrAg concentration level was closely correlated with cccDNA. Additionally, positive HBcrAg concentration level results were observed in 6 out of 13 HBsAg seroclearance patients and 20 out of 31 HBV DNA-negative patients. Moreover, the correlation between HBcrAg and cccDNA in these 31 HBV DNA-negative patients was statistically significant ($r = 0.482$, $P = 0.006$). These data suggest that serum HBcrAg concentration is well correlated with intrahepatic cccDNA level, and that the measurement of serum HBcrAg may be clinically useful for monitoring intrahepatic HBV viral status, especially in patients under treatment with nucleos(t)ide analogues. ***J. Med. Virol. 81:27–33, 2009.*** © 2008 Wiley-Liss, Inc.

KEY WORDS: HBV DNA; HBcrAg; cccDNA; HBsAg; lamivudine; entecavir

INTRODUCTION

Hepatitis B virus (HBV) is an important causative agent for liver disease such as chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Recently, several nucleos(t)ide analogues such as lamivudine [Dienstag et al., 1995], adefovir dipivoxil [Chin et al., 2001], and entecavir [Colonno et al., 2001] have been found to consistently produce rapid and dramatic decreases in viremia [Dienstag et al., 1995, 1999; Lai et al., 1998; Suzuki et al., 1999]. For the serological monitoring of chronic hepatitis patients under treatment with nucleos(t)ide analogues, improvement of alanine transaminase level, seroconversion from HBe antigen (HBeAg)-positive to anti-HBe antibody (HBeAb)-positive, and peripheral HBV DNA concentration are used as markers in chronic active hepatitis, and both HBeAg seroconversion and HBV DNA levels below the detection limit and/or of 10^5 copies/ml are commonly used as primary treatment endpoints [Lok et al., 2004]. In addition, HBV surface antigen (HBsAg) seroclearance has been linked to a good prognosis, including improvement of liver histopathology and liver function, and prolongation of survival [Arase et al., 2006], although spontaneous HBsAg seroclearance and/or remission occurred in only a small proportion of patients during the natural history of chronic HBV infections.

However, a major problem with long-term lamivudine treatment is the potential development of drug-resistance, mainly caused by the mutation of the YMDD motif of reverse transcriptase [Chayama et al., 1998]. We previously reported the efficacy of lamivudine therapy and factors associated with the emergence of resistance in chronic HBV infection in Japan [Suzuki et al., 2003].

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The measurement of the predictive serum markers of residual intrahepatic HBV DNA and/or covalently closed circular DNA (cccDNA), which is intrahepatic HBV replicated intermediate, is more important than the measurement of peripheral HBV DNA for monitoring the viral status of hepatitis patients [Sung et al., 2005]. Additionally, the amount of cccDNA in serum is reported to be higher in patients who develop YMDD mutants than in patients who do not [Yuen et al., 2005]. Recently, it was established that HBV RNA is detectable in serum and the elevation of HBV RNA is a predictor of early occurrence of viral mutation during lamivudine therapy [Rokuhara et al., 2006; Hatakeyama et al., 2007]. However, these HBV DNA, HBV RNA, or cccDNA detection assay methods remain complicated and difficult to perform. Therefore, simple methods of viral status evaluation are required for routine assays rather than for nucleic acid assays.

Recently, a new assay was performed for the detection of hepatitis B core-related antigen (HBcrAg) consisting of HBV core antigen (HBcAg), HBeAg, and 22 kDa precore protein (p22cr) coded with precore/core gene [Kimura et al., 2002, 2005]. p22cr is a precore protein from amino acid −28 to at least amino acid 150, containing an uncleaved signal sequence and lacking the C-terminal arginine-rich domain. p22cr is found in empty and HBV DNA negative virus particles; the production of empty particles is not dependent on the formation of HBV DNA [Kimura et al., 2005]. Several reports indicate that the concentration of serum HBcrAg is closely correlated with peripheral HBV DNA in untreated patients [Rokuhara et al., 2003; Tanaka et al., 2006]. Additionally, HBcrAg is considered as a prospective marker of the appearance of drug-resistant HBV mutants and of the identification of patients with low risk of HBV reactivation after discontinuation of lamivudine administration, while peripheral HBV DNA does not qualify as a prospective marker in these patients [Rokuhara et al., 2005; Shinkai et al., 2006; Tanaka et al., 2006; Matsumoto et al., 2007]. The relationship between HBcrAg and intrahepatic cccDNA levels has not yet been clarified.

In this study, we examined the correlation between HBcrAg and viral status by the analysis of HBeAg, HBsAg, peripheral HBV DNA, and intrahepatic cccDNA in patients with chronic hepatitis B.

MATERIALS AND METHODS

Patients and Samples

Serum samples and biopsy specimens were obtained from 57 chronic hepatitis B patients at Toranomon Hospital under informed consent. The median age of patients was 49 (range, 25–71 years). Out of 57 patients, 28 underwent nucleos(t)ide analogue administration (17 patients of lamivudine, 7 patients of both lamivudine and adefovir dipivoxil, 4 patients of entecavir), and 13 were HBsAg-negative/HBs-seroclearance patients with more than 12 months of being HBsAg-positive before HBs-seroclearance.

Routine Laboratory Tests

HBsAg, HBeAg, and HBeAb were routinely measured by the commercially available Chemiluminescent Enzyme Immunoassay (CLEIA) (Lumipulse System, Fujirebio, Inc., Tokyo, Japan).

HBcrAg Test

Serum HBcrAg was measured by CLEIA HBcrAg assay kit (Fujirebio, Inc.) with a fully automated analyzer system (Lumipulse System, Fujirebio, Inc.). Briefly, 150 μl of serum was incubated with 150 μl of pretreatment solution containing 15% sodium dodecyl sulfate 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 p22cr. After 10 min incubation at 37°C and washing, further incubation was done for 10 min at 37°C with alkaline phosphatase conjugated with two kinds of monoclonal antibodies (HB91 and HB110) against denatured HBcAg, HBeAg, and p22cr. After washing, 200 μl of substrate solution [AMPPD: 3-(2′-spiroadamantan)-4-methoxy-4- (3″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) was added to the test cartridge which was then incubated for 5 min at 37°C. The relative chemiluminescence intensity was measured, and HBcrAg concentration was calculated by a standard curve generated using recombinant pro-HBeAg (amino acids: −10 to 183 of precore/core gene product). HBcrAg concentration was expressed as units/ml (U/ml), which is defined as the immunoreactivity of 10 fg/ml of recombinant pro-HBeAg. In this study, HBcrAg value was expressed as log U/ml, and the cut-off value was set at 3.0 log U/ml. For the statistical analysis, HBcrAg-negative cases were calculated as 3.0 log U/ml.

HBV DNA Assay

HBV DNA in serum was measured by polymerase chain reaction (PCR) assay kit (Amplicor HBV monitor test, Roche Molecular Systems, Inc., Branchburg, NJ). Values under or over the detection range were calculated as 2.6 log copies/ml or as 7.6 log copies/ml, respectively.

Measurement of cccDNA

Liver biopsy specimens were taken and stored at −80°C before DNA extraction. HBV DNA was extracted using QIAamp DNA Mini Kit (Qiagen KK, Tokyo, Japan). The concentration of purified DNA was based on absorbance at 260 nm. For this study, two oligonucleotide primers, cccF2 (5′-cgtctgtgccttctcatctga-3′, nucleotides 1,424–1,444), cccR4 (5′-gcacagcttggaggcttgaa-3′, nucleotides 1,755–1,737), and a probe cccP2 (5′-VIC-accaatttatgcctacag-MGB-3′, nucleotides 1,672–1,655), were designed using Primer Express™ software (Applied Biosystems, Foster City, CA) 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 PCR 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 [Mason et al., 1998]. Twenty-five microliters of the extracted DNA (0.5 μg) were 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 was activated at 95°C for 10 min. The subsequent PCR conditions consisted of 45 cycles of denaturation at 95°C for 15 sec, and annealing and extension at 60°C for 90 sec per cycle (SRL, Inc., Tokyo, Japan).

Statistical Analysis

The statistical analysis of the correlation data between serum HBcrAg, HBsAg, HBV DNA, and/or cccDNA was performed by SPSS software (version 14.0J, SPSS Japan Inc., Tokyo, Japan), and the statistical significance between the two sides was taken as *P*-value lower than 0.05.

RESULTS

Serological and Genetic Assay Results

We classified the 57 patients according to assay results of HBsAg, HBeAg, serum HBV DNA, intrahepatic HBV cccDNA, and serum HBcrAg. Positive results were observed with all 57 patients in the cccDNA assay, in 44 patients with the HBsAg test, in 16 patients with the HBeAg test, in 26 patients with the HBV DNA assay, and in 42 patients with the HBcrAg assay (Table I). Among the 13 patients with negative results with HBsAg, HBeAg, and HBV DNA but positive results with cccDNA assay, six patients showed HBcrAg-positive results, although the serum HBcrAg concentration value was low (mean value ± standard deviation: 3.23 ± 0.27 log U/ml) in comparison to that of the group with positive results with HBsAg, HBeAg, and HBV DNA (6.91 ± 1.06 log U/ml) tests. Among the 28 patients with HBsAg-positive but HBeAg-negative results, 20 patients were HBcrAg-positive and 10 out of these 20 patients showed negative HBV DNA assay results.

Next, assay results were analyzed according to presence/absence of nucleos(t)ide analogue treatment, HBsAg-positive/negative results, and HBeAg-positive/negative results by the combination with treated/untreated subgroups (Table II). When patients were classified into two groups, namely 28 patients treated with nucleos(t)ide analogues and 29 untreated patients, no difference was observed in average mean value of HBcrAg, HBV cccDNA, and HBV DNA (data not shown). However, since the 13 HBsAg-negative patients were clinically stable, we further analyzed the 44 HBsAg-positive patients by grouping them according to pres-

TABLE I. Summary of HBcrAg Concentration in Positive and/or Negative Patients by HBsAg, HBeAg, HBV DNA, and HBcrAg Assay

All cases		Classified by assay results with:							
		HBsAg		HBeAg		HBV DNA		HBcrAg	
Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)	Results (number)	HBcrAg (±SD)
All (N = 57)	4.61 (1.64)	Positive (N = 44)	5.05 (1.62)	Positive (N = 16)	6.53 (1.14)	Positive (N = 12)	6.91 (1.06)	Positive (N = 12)	6.91 (1.06)
								Negative (N = 0)	
						Negative (N = 4)	5.40 (0.38)	Positive (N = 4)	5.40 (0.38)
								Negative (N = 0)	
				Negative (N = 28)	4.20 (1.18)	Positive (N = 14)	4.29 (1.20)	Positive (N = 10)	4.81 (1.02)
								Negative (N = 4)	<3.00
						Negative (N = 14)	4.11 (1.20)	Positive (N = 10)	4.55 (1.14)
								Negative (N = 4)	<3.00
		Negative (N = 13)	3.11 (0.21)	Positive (N = 0)		Positive Negative (N = 0)		Positive Negative (N = 0)	
				Negative (N = 13)	3.11 (0.21)	Positive (N = 0)		Positive Negative (N = 0)	
						Negative (N = 13)	3.11 (0.21)	Positive (N = 6)	3.23 (0.27)
								Negative (N = 7)	<3.00

Mean value: log U/ml ± Standard deviation.

TABLE II. Classification of HBcrAg, cccDNA and HBV DNA Assay Results According to Presence/Absence of Treatment, HBeAg Test and HBsAg Test

			HBcrAg (log U/ml)		cccDNA (log copy/mg)		HBV DNA (log copy/ml)	
Item	Category	N	Mean (SD)	*P* value	Mean (SD)	*P* value	Mean (SD)	*P* value
Unclassified	All	57	4.61 (1.64)		4.25 (0.91)		3.67 (1.59)	
Treatment[a]	With	26	4.77 (1.49)	NS[c]	4.41 (0.68)	NS[c]	3.20 (1.08)	<0.001
	Without	18	5.45 (1.75)		4.54 (1.09)		5.13 (1.78)	
HBsAg	Positive							
	Total	44	5.05 (1.62)		4.46 (0.87)		3.99 (1.69)	
	Negative			<0.001		0.001		<0.001
	Total	13	3.11 (0.21)		3.52 (0.68)		2.60 (0.00)	
HBeAg[b]	Positive							
	Total	16	6.53 (1.14)		4.88 (1.06)		5.17 (1.92)	
	Negative			0.001		0.015		0.002
	Total	28	4.20 (1.18)		4.23 (0.64)		3.32 (1.09)	
	Positive							
	Treated	8	6.21 (1.09)	NS[c]	4.76 (0.95)	NS[c]	3.83 (1.64)	0.005
	Untreated	8	6.85 (1.18)		5.00 (1.21)		6.51 (1.02)	
	Negative							
	Treated	18	4.13 (1.16)	NS[c]	4.25 (0.49)	NS[c]	2.93 (0.57)	0.016
	Untreated	10	4.33 (1.26)		4.18 (0.88)		4.02 (1.45)	

[a]In the with/without treatment group, 44 HBsAg-positive patients were analyzed.
[b]HBsAg-positive 44 patients were further separated into HBeAg-positive and HBeAg–negative groups. In addition, these groups were further separated into treated and untreated groups; the mean value of each assay was calculated and a statistical analysis was done.
[c]NS: statistically not significant.

ence/absence of treatment of nucleos(t)ide analogues (Table II). HBcrAg concentration was 4.77 ± 1.49 log U/ml in 26 treated patients and 5.45 ± 1.75 log U/ml in 18 untreated patients (not statistically significant). Similar results in cccDNA levels were observed in both treated and untreated groups (4.41 ± 0.68 log copy/μg and 4.54 ± 1.09 log copy/μg, not statistically significant). In contrast, lower HBV DNA was observed in the treated group (3.20 ± 1.08 log copy/ml) as compared with the untreated group (5.13 ± 1.78 log copy/ml, $P < 0.001$).

Statistically significant results were observed under grouping according to HBsAg assay results; namely, HBcrAg was 5.05 ± 1.62 log U/ml in the HBsAg-positive group and 3.11 ± 0.21 log U/ml in the HBsAg-negative group ($P < 0.001$), HBV cccDNA was 4.46 ± 0.87 log copies/μg in the HBsAg-positive group and 3.52 ± 0.68 log copies/μg in the HBsAg-negative group ($P < 0.001$), HBV DNA was 3.99 ± 1.69 log copies/ml in the HBsAg-positive group and <2.60 log copies/ml in the HBsAg-negative group ($P < 0.001$).

Similar results were observed under grouping according to HBeAg results. In this analysis, HBsAg-negative patients were omitted because HBeAg-negative patients included both HBsAg-positive/negative patients, whereas all HBeAg-positive patients were HBsAg-positive. The mean values of HBcrAg, HBV cccDNA, and HBV DNA in the HBeAg-positive group were higher than those of the HBeAg-negative group; namely, HBcrAg concentration was 6.53 ± 1.14 log U/ml in the HBeAg-positive group and 4.20 ± 1.18 log U/ml in the HBeAg-negative group ($P < 0.001$), cccDNA was 4.88 ± 1.06 log copy/μg in the HBeAg-positive group and 4.23 ± 0.64 log copies/μg in the HBeAg-negative group ($P = 0.015$), HBV DNA was 5.17 ± 1.92 log copies/ml in the HBeAg-positive group and 3.32 ± 1.09 log copies/ml in the HBeAg-negative group ($P = 0.002$). When the HBeAg-positive group was further separated into treated and untreated groups, these three markers in eight treated patients were lower than in eight untreated patients; namely, HBcrAg (6.21 ± 1.09 log U/ml vs. 6.85 ± 1.18 log U/ml), cccDNA (4.76 ± 0.95 log copy/μg vs. 5.00 ± 1.21 log copy/μg), and HBV DNA (3.83 ± 1.64 log copy/ml vs. 6.51 ± 1.02 log copy/ml), although HBcrAg and cccDNA values between the treated and untreated groups were not statistically significant. In the HBeAg-negative group, statistically significant ($P = 0.016$) lower values of HBV DNA but not of HBcrAg and cccDNA were observed in 18 treated patients by comparison with 10 untreated patients. The HBV DNA level of 8 out of 10 untreated patients was less than 5 log copies/ml. These patients underwent a liver biopsy for progress follow-up. Therefore, there was a relatively small difference in HBV DNA level between treated and untreated patients.

Correlation Between HBcrAg, HBV DNA and/or cccDNA

The correlation between HBcrAg, HBV DNA, and/or cccDNA in all 57 patients was summarized in Figure 1. A statistically significant positive correlation was observed in all analyses, namely HBcrAg versus HBV cccDNA (Fig. 1a, $r = 0.692$, $P < 0.001$), HBcrAg versus HBV DNA (Fig. 1b, $r = 0.713$, $P < 0.001$), and HBV cccDNA versus HBV DNA (Fig. 1c, $r = 0.637$, $P < 0.001$).

Next, HBcrAg concentration in 31 HBV DNA-negative patients was measured; 20 patients showed levels greater than 3.0 log U/ml. A statistically significant correlation between HBcrAg and cccDNA in these 31 patients was observed (Fig. 2, $r = 0.482$, $P = 0.006$),

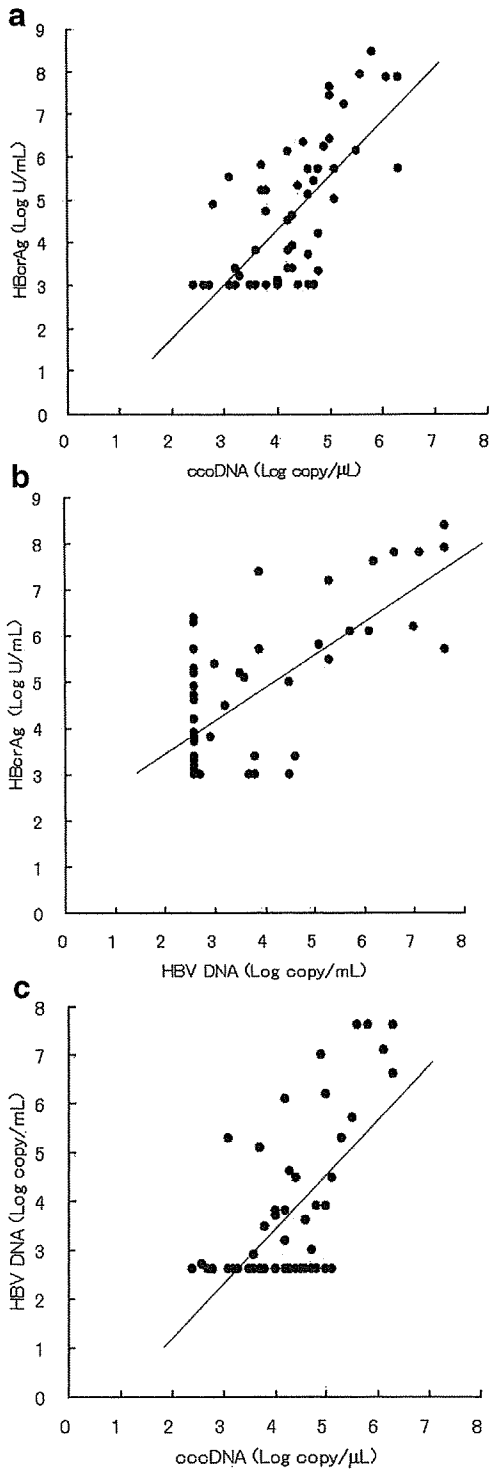

Fig. 1. Correlation between serum HBcrAg and intrahepatic HBV cccDNA in 57 patients with chronic hepatitis B (**a**: $y = 1.25 \times -0.69$, $r = 0.692$, $P < 0.001$), HBcrAg and serum HBV DNA (**b**: $y = 0.74 \times +1.91$, $r = 0.713$, $P < 0.001$), and serum HBV DNA and intrahepatic cccDNA (**c**: $y = 1.11 \times -1.05$, $r = 0.637$, $P < 0.001$). Straight lines indicate the correlation between each other.

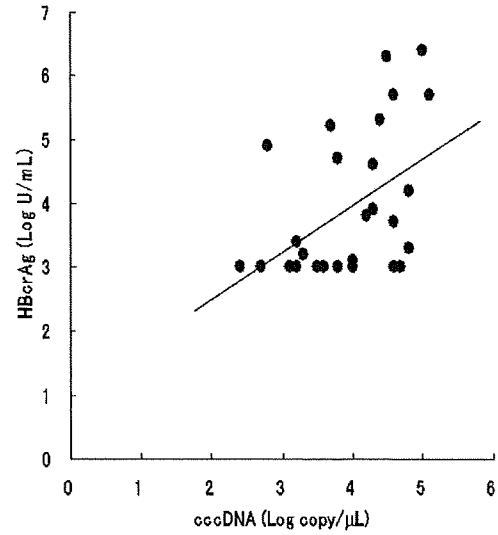

Fig. 2. Correlation between HBcrAg and cccDNA in 31 HBV DNA negative patients ($y = 0.73 \times +1.00$, $r = 0.482$, $P = 0.006$).

suggesting that HBcrAg measurement may be a useful marker in HBV DNA-negative patients as a substitute for cccDNA assay.

When 44 HBsAg positive patients were grouped according to whether they were HBeAg-positive or HBeAg-negative, HBcrAg concentration was correlated with cccDNA in both 16 HBeAg-positive patients (Fig. 3a, $r = 0.687$, $P = 0.003$) and 28 HBeAg-negative patients (Fig. 3a, $r = 0.542$, $P = 0.003$), and with HBV DNA in the HBeAg-positive (Fig. 3b, $r = 0.681$, $P = 0.004$) but not in the HBeAg-negative group (Fig. 3b, $r = 0.311$, $P = 0.107$). A positive correlation between HBV DNA and cccDNA was also observed in both the HBeAg-positive group (Fig. 3c, $r = 0.588$, $P = 0.017$) and the HBeAg-negative group (Fig. 3c, $r = 0.442$, $P = 0.018$).

DISCUSSION

Nucleos(t)ide analogues have a suppressive effect on the transcription of pregenomic RNA, and the administration of these agents can induce a rapid and dramatic decrease in peripheral HBV DNA, seroclearance of HBeAg, and remission of chronic hepatitis B [Dienstag et al., 1995, 1999; Lai et al., 1998]. However, these nucleos(t)ide analogues are unable to induce an adequate and complete elimination of HBV. Therefore, the measurement of intrahepatic HBV DNA and/or HBV cccDNA is important for monitoring the viral status of hepatitis patients [Sung et al., 2005], although these assays involve the physical stress of needle biopsy.

Several reports indicate that the level of HBcrAg, which is a complex of HBeAg, HBcAg, and p22cr coding precore/core gene [Kimura et al., 2002, 2005], reflects the natural course of viral loads in patients under treatment with nucleos(t)ide analogues, and that the reduction rate of HBcrAg is slower than that of serum

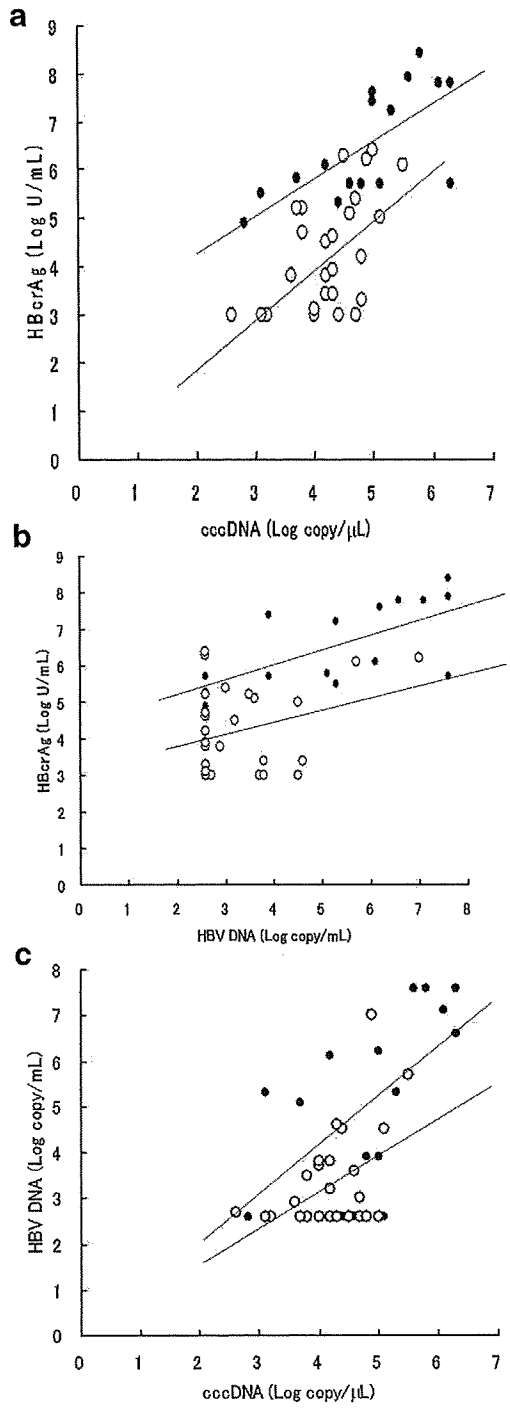

Fig. 3. Correlation between HBcrAg, cccDNA and HBV DNA in 44 HBsAg-positive with 16 HBeAg-positive and 28 HBeAg-negative patients. **a**: Correlation between HBcrAg and cccDNA (HBeAg-positive cases as closed circle; $y = 0.74 \times +2.91$, $r = 0.687$, $P = 0.003$, HBeAg-negative cases as open circle; $y = 1.00 \times -0.02$, $r = 0.542$, $P = 0.003$). **b**: Correlation between HBcrAg and serum HBV DNA (HBeAg-positive patients as closed circle; $y = 0.41 \times +4.43$, $r = 0.681$, $P = 0.004$, HBeAg-negative patients as open circle; $y = 0.34 \times +3.09$, $r = 0.311$, $P = 0.107$). **c**: Correlation between serum HBV DNA and intrahepatic cccDNA (HBeAg-positive cases as closed circle; $y = 1.07 \times -0.03$, $r = 0.588$, $P = 0.017$, HBeAg-negative patients as open circle; $y = 0.76 \times +0.12$, $r = 0.442$, $P = 0.018$).

HBV DNA [Rokuhara et al., 2003; Tanaka et al., 2006]. Similar results were observed in patients infected with genotypes B and C of HBV [Rokuhara et al., 2005]. This phenomenon may be explained by the fact that the production of HBcrAg depends on the transcription of mRNA from cccDNA, and that cccDNA still remains in high levels after treatment with these nucleos(t)ide analogues. Therefore, several reports suggest that HBcrAg may be a predicting marker for relapse after cessation of lamivudine therapy in chronic HBV infection [Shinkai et al., 2006; Matsumoto et al., 2007] and that it may also help identify patients who are at low risk of lamivudine resistance [Tanaka et al., 2006].

In this study, we analyzed the correlation between HBcrAg and several HBV markers, especially HBV cccDNA. Results indicated a good correlation of HBcrAg against serum HBV DNA and intrahepatic HBV cccDNA (Fig. 1). In addition, 20 out of 31 HBV DNA-negative patients showed more than 3.0 log U/ml in HBcrAg. All of these 20 patients were also cccDNA-positive, and there was a positive correlation between HBcrAg and cccDNA levels, although HBcrAg was negative in 11 patients (Fig. 2). The production of HBcrAg is considered to depend on the transcription of mRNA from intrahepatic cccDNA. Our data showed that serum HBcrAg may reflect intrahepatic cccDNA. Therefore, measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring chronic hepatitis B patients. Recently, the acquisition of de novo HBV-related hepatitis after liver transplantation has become an important cause of morbidity and mortality. Moreover, de novo HBV-related hepatitis has been reported in patients after hematopoietic stem cell transplantation and cytotoxic chemotherapy treatment [Dhedin et al., 1998; Hui et al., 2006]. Therefore, HBcrAg may be a useful marker of occult HBV infection in these patients.

Several reports indicate that HBsAg seroclearance confers favorable long-term outcomes in patients without hepatocellular carcinoma or decompensated liver cirrhosis [Arase et al., 2006; Kobayashi et al., 2006]. However, studies show that intrahepatic HBV DNA still remains in HBsAg seroclearance cases [Arase et al., 2006], and that 10–20% of patients have 50–100 copies/ml of serum HBV DNA for 5 and 10 years after seroclearance of HBsAg [Arase et al., 2007]. In this study, 6 out of 13 patients with HBsAg seroclearance showed HBcrAg-positive results (3.23 ± 0.27 log U/ml), and all 13 patients remained cccDNA-positive (3.52 ± 0.68 log copy/μg). These data suggest that HBV remains present for a prolonged period after HBsAg seroclearance, further studies are thus necessary to clarify the mechanism of HBcrAg production and/or the regulation of mRNA in chronic hepatitis with HBsAg seroclearance.

Meanwhile, positive correlations between HBV DNA and HBcrAg were not observed in the HBsAg-positive and HBeAg-negative group (Fig. 3b), although HBcrAg concentration was correlated with cccDNA in HBeAg-negative patients (Fig. 3a). This finding shows that

measurement of HBcrAg as a substitute for cccDNA may be useful for monitoring patients in HBsAg-positive and HBeAg-negative groups.

In conclusion, serum HBcrAg concentration appears to be well correlated with intrahepatic cccDNA level, and the measurement of serum HBcrAg as substitute for cccDNA and/or serum HBV DNA may be clinically useful for the monitoring of intrahepatic HBV viral status.

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CLINICAL STUDIES

Predictive factors of advanced recurrence after curative resection of small hepatocellular carcinoma

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Abstract

Background: The tumour recurrence rate after resection is still high even in patients with small hepatocellular carcinoma (HCC). The advanced patterns of recurrence occasionally occur after resection. In this study, we analysed the clinical and histological characteristics of small HCC and evaluated the predictive factors of advanced tumour recurrence. *Methods:* One hundred and sixty-five patients underwent resection of small HCC measuring 3 cm or less in greatest dimension. Patterns of tumour recurrences were classified into advanced recurrence and minor recurrence based on size, number, vascular invasion and extrahepatic metastasis of recurrent tumour. We created a simple index to closely evaluate the malignant potential of small HCC, named α-foetoprotein–size ratio index (ASRI). *Results:* Overall tumour recurrence was significantly associated with tumour multiplicity ($P < 0.001$) and ASRI ($P = 0.001$). Tumour multiplicity, ASRI and tumour differentiation were independent and significant predictive factors of advanced recurrences. The overall survival rates were lower in the advanced recurrence group than the minor recurrence or the no recurrence group. *Conclusions:* Patients with advanced recurrences have a poor prognosis, although they have undergone curative resection of small HCC. On the other hand, patients with minor recurrences have a relatively good prognosis. ASRI was a useful index to predict advanced recurrence after curative resection of small HCC. The therapeutic management to prevent advanced recurrences is needed.

Hepatocellular carcinoma (HCC) is one of the most common neoplasms in Africa and Asia, including Japan. Routine checkups are performed in patients with hepatitis or cirrhosis who constitute a significant high-risk group for HCC (1–3). Recently, technological advances in ultrasonography (US), computed tomography (CT) and magnetic resonance imaging have helped in the detection of small HCC during follow-up periods of chronic liver disease (4, 5). Moreover, resection of HCC has become safe in cirrhotic patients due to progress in surgical techniques, and perioperative management has contributed to very low operative mortality. However, the tumour recurrence rate after resection is still high even in patients with small HCCs (6–10). Recurrences in the remnant liver can occur based on two characteristics of HCC: intrahepatic metastasis from the primary tumour and de novo multicentric carcinogenicity (11–13).

Tumour status at the time of recurrence is important to improve prognosis because tumour recurrence rates after curative resection are high. The advanced patterns of recurrence occasionally occur as follows: widespread recurrence, a number of recurrent tumours, large recurrent tumour, involving vascular invasion and extrahepatic metastasis, despite curative resection (14–16). Because the therapeutic approach for recurrent tumours is limited, these cases have a poor prognosis. Therefore, it is important to pick up patients who are likely to have these advanced recurrence, and to develop effective adjuvant therapy. In the present study, we examined the clinical features of small HCC, and identified the factors associated with tumour recurrence, especially advanced recurrence and prognosis after curative resection of small HCCs using clinical data and results of histopathological examination. Furthermore, we created a simple index to closely evaluate the malignant potential of small HCC and evaluated the usefulness of this index as a predictor of recurrence of HCC after curative resection.

Patients and methods

Patients

Medical records of patients who were hospitalized at Toranomon Hospital from 1995 to 2005 were reviewed retrospectively. HCC was diagnosed by detailed imaging or histopathological examination. A total of 251 consecutive patients with tumours underwent resection as the initial therapy for HCC, and 165 of these patients were found to have HCC measuring $\leq$ 3 cm (greatest dimension) and were eligible for inclusion in this study. These 165 patients (127 men and 38 women; median age 61 years; range, 38–73 years) had chronic hepatitis or cirrhosis. Hepatitis B virus (HBV) surface antigen was positive in 33, anti-hepatitis C virus (HCV) was positive in 127, but neither of them was positive in eight. Table 1 lists the clinical characteristics of the 165 patients before hepatectomy. Of these, 125 patients (75.6%) were classified as grade A according to Child–Pugh classification. The median value for the indocyanine green retention rate at 15 min was 24%, and the median values for serum albumin, bilirubin, aspartic transaminase (AST), α-foetoprotein (AFP) concentration and platelet counts were 3.7 g/dl, 1.0 mg/dl, 44 IU/L, 26 ng/ml and $10.8 \times 10^4/mm^3$ respectively.

Among 165 patients, 26 patients (15.8%) had multiple tumours before resection. We conducted percutaneous ablation therapy, including ethanol injection, microwave coagulation

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Table 1. Clinical characteristics of 165 patients before hepatic resection

Variables	$n = 165$
Age	62 (38–80)*
Gender (male:female)	127:38
Hepatitis B surface antigen-positive	46 (27.9%)
Anti-hepatitis C virus-positive	109 (66.1%)
Child–Pugh classification (A:B:C)	125:38:1
Serum albumin (g/dl)	3.6 (2.6–4.6)*
Serum bilirubin (mg/dl)	1.0 (0.3–2.7)*
Aspartate transaminase (IU/L)	44 (12–386)*
Prothorombin time (%)	90.8 (58.9–112.8)*
ICG R15 (%)	21 (8–68)*
Platelet count (10^4/mm^3)	12.6 (3.9–26.0)*
α-foetoprotein (ng/ml)	23 (1–7960)*
Des-γ-carboxy prothorombin (mAU/ml)	22 (< 10–1650)*
Tumour size (mm)	20 (7–30)*
Tumour number (solitary:multiple)	139:26
Vascularity positive	153 (92.7%)
ASRI	1.2 (0.03–345)*

*Values are medians (range).
ASRI, α-foetoprotein–size ratio index = AFP (ng/ml)/tumour size (mm); ICG R15, indocyanine green retention test at 15 min.

and radiofrequency ablation, for another tumour before surgery if another tumour existed in a lobe distant from the resected tumour. The term 'curative resection' indicated that no tumours were left in the remnant liver irrespective of the width of margin around the tumour; this was confirmed using (i) intra-operative US and (ii) combined US and dynamic CT conducted after 1 month of surgery.

This study was conducted in accordance with the guidelines of the Declaration of Helsinki and its subsequent amendments, and informed consent was obtained from every patient. This study was approved by the Local Ethics Committee of Toranomon Hospital.

Follow-up and recurrence of hepatocellular carcinoma

Patients were followed up on a monthly or a bi-monthly basis after surgery by monitoring AFP and other biochemical data, and conducting US or helical dynamic CT every 3 months. The median observation period for the entire patient cohort was 6.0 years, with a range of 0.3–16.4 years. Recurrence of HCC was diagnosed by typical hypervascular characteristics on angiography and/or histological examination with fine needle biopsy specimens, in addition to certain features of CT and US.

The modes of cancer recurrence were classified into two categories: (i) advanced recurrence and (ii) minor recurrence. The patterns of recurrence were morphologically judged from the images of CT and angiography, and from histopathological findings. The pattern of recurrent tumour number > 3, tumour size > 3 cm, involving vascular invasion and/or extrahepatic metastasis was defined as advanced recurrence. The recurrent pattern, except for those described above, was defined as minor recurrence.

Imaging analysis

Ultrasonography or helical dynamic CT was carried out every 3 months for follow-up and examined for a change in imaging findings. Dynamic CT scans were performed using a single-detector helical CT scanner (Hi-Speed advantage SG; GE Yokogawa Medical Systems, Tokyo, Japan). The radiological studies included intra-arterial digital subtraction angiography (celiac and mesenteric angiography) and selective angiography of the common hepatic artery. CT arterial portography (CT-AP) and CT hepatic angiography (CT-HA) were carried out in almost all patients before surgery. HCC was diagnosed by typical hypervascular characteristics on angiography and/or CT-HA, and hypo-attenuation on CT-AP. If hepatic nodules showed iso-hypo-attenuation on CT-HA and iso-hypo-attenuation on CT-AP, histological examination was carried out with fine needle biopsy specimens before surgery.

Table 2. Pathological characteristics of small hepatocellular carcinoma

Variables	$n = 165$
Tumour differentiation (early:well:moderately:poorly)	11:32:100:22
Growth type (Eg:Ig)	138:27
Capsular formation	99 (60.0%)
Capsular infiltration	52 (31.5%)
Septum formation	42 (25.5%)
Portal vein invasion	26 (15.8%)
Intrahepatic extent of tumour	5 (3.0%)
Presence of cirrhosis	114 (69.1%)

Eg, expansive growth (well-demarcated border); Ig, infiltrative growth (poorly demarcated border).

Histopathological examination

Macroscopic and microscopic examinations were performed according to the classification of the Liver Cancer Study Group of Japan (17). All resected specimens were analysed histopathologically for tumour size, growth type, tumour differentiation, capsular formation, portal vein invasion, satellite nodules and fibrosis staging of surrounding liver. The tumour characteristics are summarized in Table 2. We categorized well-differentiated HCC that had histological features of the early stage into early HCC. Early HCC was defined as follows: macroscopically, the tumours had an indistinct margin that replaced the liver cell cords at the tumour–non-tumour boundary; microscopically, increased cell density with an increased nuclear to cytoplasm ratio and an irregular thin-trabecular pattern, and the portal tracts were involved inside the tumours together with tumour cell invasion into the portal tracts (18–20).

α-foetoprotein–size ratio index

In this study, there were patients with very high AFP levels regardless of the cohort of small HCC measuring 3 cm or less in greatest dimension. We hypothesized that HCCs with high AFP levels had more malignant potential than those with low AFP levels if each tumour size was equal. And so, we created a simple index to closely evaluate the malignant potential of small HCC, named the AFP–size ratio index (ASRI). The numerical formula of ASRI was defined as follows: ASRI = AFP levels (ng/ml)/tumour size (mm). For example, the calculated value of ASRI of HCC, with tumour size = 20 mm and AFP levels = 400 ng/ml, is 20.

Statistical analysis

Standard statistical measures and procedures were used. We used the χ^2-test to assess the significant association of risk

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factors with tumour recurrence after resection. All factors found to be at least marginally associated with recurrence ($P < 0.15$) were tested by multivariate analysis. Independent factors, associated with the recurrence of HCC and prognosis, were calculated using stepwise Cox regression analysis. The χ^2-test was used to analyse differences between the clinical characteristics of HCC and the patterns of tumour recurrences. The cumulative overall survival rates after resection of small HCC 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 using the SPSS software, version 11.0 (Chicago, IL, USA).

Results

Factors associated with tumour recurrences

Univariate analysis showed that tumour recurrence was significantly associated with tumour multiplicity ($P < 0.001$), ASRI ≥ 20 ($P = 0.004$), AFP levels ≥ 1000 ng/ml ($P = 0.024$), portal vein invasion ($P = 0.035$) and serum albumin levels ≥ 3.5 g/dl ($P = 0.041$), and marginally significantly with HCV positivity ($P = 0.058$), HBV negativity ($P = 0.072$), hypervascularity of tumour ($P = 0.076$) and serum AST levels ≥ 50 IU/L ($P = 0.088$) (Table 3). Because these variables were associated, multivariate analysis was performed using the nine variables mentioned above in the model (Table 4a). The following two variables were significantly associated with overall tumour recurrence: tumour multiplicity [hazard ratio (HR) 3.06, 95% confidence interval (CI): 1.84–5.10; $P < 0.001$], ASRI ≥ 20 (HR 2.42, 95% CI: 1.41–4.18, $P = 0.001$). To evaluate risk factors except for tumour multiplicity, subgroup analysis was conducted in solitary tumour cases (Table 4b). Independent risk factors affecting the overall recurrence of HCC were the presence of portal vein invasion (HR 2.35, 95% CI: 1.31–4.20, $P = 0.004$), ASRI ≥ 20 (HR 2.23, 95% CI: 1.19–4.18, $P = 0.013$) and serum albumin < 3.5 g/dl (HR 1.74, 95% CI: 1.05–2.88, $P = 0.030$).

Predictive factors of advanced recurrences after curative resection

Tumour recurrence was diagnosed in 102 (61.8%) of the 165 patients, with a median interval of 2.77 years after curative resection. Of these, 22 (13.3%) were categorized into advanced recurrence, 80 (48.4%) were minor recurrence and the remaining 63 (38.1%) were no recurrence. The median interval to recurrence after resection was 1.82 years in the minor recurrence group and 1.01 years in the advanced recurrence group respectively. Univariate analysis showed that advanced recurrence was significantly associated with the following four factors: poorly differentiation of tumour ($P < 0.001$), ASRI ≥ 20 ($P = 0.005$), tumour multiplicity ($P = 0.017$) and AFP levels ≥ 1000 ng/ml ($P = 0.025$) (Table 5). Multivariate analysis by the Cox model was performed using the four variables mentioned above. Predictive factors of advanced recurrences after curative resection were tumour multiplicity (HR 5.65, 95% CI: 1.77–18.1, $P = 0.003$), ASRI ≥ 20 (HR 4.04, 95% CI: 1.16–14.1, $P = 0.028$) and poor differentiation of tumour (HR 2.70, 95% CI: 1.51–4.82, $P = 0.001$) (Table 6).

We compared values of ASRI by patterns of recurrences (Fig. 1). The median values of ASRI were 0.68 (minimum: 0.07–maximum: 73.0) in the no recurrence group, 1.64 (0.06–344) in the minor recurrence group and 3.28 (0.03–318) in the advanced recurrence group respectively. The values of ASRI were marginally significantly higher in the minor recurrence and the advanced recurrence group than in the no recurrence group. However, there was no significance of ASRI values stratified by tumour number.

Furthermore, we categorized the following three subgroups into the advanced recurrence group: tumour number > 3, or tumour size > 3 cm without vascular invasion and extrahepatic metastasis (multi/large nodular recurrence group), recurrent tumour with vascular invasion (vascular invasion group) and

Table 3. Factors associated with overall recurrence of small hepatocellular carcinoma by univariate analysis

Factors	Hazard ratio (95% CI)	*P*
Age (≥ 65 vs. < 65 years)	0.79 (0.52–1.22)	0.288
Gender (female vs. male)	0.78 (0.48–1.26)	0.316
HBV (negative vs. positive)	1.52 (0.96–2.41)	0.072
HCV (positive vs. negative)	1.53 (0.99–2.36)	0.058
Serum albumin (< 3.5 vs. ≥ 3.5 g/dl)	1.53 (1.02–2.31)	0.041
Serum bilirubin (≥ 1.5 vs. < 1.5 mg/dl)	1.11 (0.62–2.00)	0.713
AST levels (≥ 50 vs. ≥ 50 IU/L)	1.41 (0.95–2.10)	0.088
Prothorombin time (≥ 70 vs. $< 70\%$)	0.67 (0.31–1.45)	0.311
ICG R 15 (≥ 30 vs. $< 30\%$)	1.37 (0.89–2.12)	0.158
count ($\geq 10^5$ vs. $< 10^5$/mm^3)	0.81 (0.54–1.22)	0.304
AFP levels (≥ 1000 vs. < 1000 ng/ml)	2.01 (1.10–3.67)	0.024
ASRI (≥ 20 vs. < 20)	2.16 (1.28–3.64)	0.004
DCP levels (≥ 100 vs. < 100 mAU/ml)	1.19 (0.70–2.04)	0.517
Fibrosis stage (F4 vs. F1, 2, 3)	1.09 (0.72–1.66)	0.681
Tumour size (≥ 21 vs. < 21 mm)	1.088 (0.73–1.63)	0.680
Tumour number (multiple vs. solitary)	2.85 (1.74–4.65)	< 0.001
Vascularity (positive vs. negative)	2.48 (0.91–6.76)	0.076
Tumour differentiation (poorly vs. early, well, moderately)	1.15 (0.87–1.51)	0.333
Eg	1.00 (0.60–1.68)	0.987
Capsular formation	1.01 (0.68–1.52)	0.948
Infiltration to capsular	1.39 (0.92–2.10)	0.121
Septum formation	0.99 (0.63–1.56)	0.969
Portal vein invasion	1.70 (1.04–2.78)	0.035
Intrahepatic extent of tumour	1.57 (0.58–4.26)	0.380

AFP, α-foetoprotein; ASRI, α-foetoprotein–size ratio index; AST, aspartic transaminase; DCP, des-γ-carboxy prothorombin; Eg, expansive growth (well-demarcated border); HBV, hepatitis B virus; HCV, hepatitis C virus; ICG R15, indocyanine green retention test at 15 min.

Table 4a. Independent risk factors affecting the overall recurrence of hepatocellular carcinoma after curative resection of small hepatocellular carcinoma by multivariate analysis

Factors	Category	Hazard ratio (95% CI)	*P* value
Tumour number	1: solitary	1	
	2: multiple	3.06 (1.84–5.10)	< 0.001
ASRI	1: < 20	1	
	2: ≥20	2.42 (1.41–4.18)	0.001

ASRI, α-foetoprotein–size ratio index; CI, confidence interval.

Furthermore, we categorized the following three subgroups into the advanced recurrence group: tumour number > 3, or tumour size > 3 cm without vascular invasion and extrahepatic metastasis (multi/large nodular recurrence group), recurrent tumour with vascular invasion (vascular invasion group) and

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Table 4b. Independent risk factors affecting the overall recurrence of hepatocellular carcinoma after curative resection of small hepatocellular carcinoma by multivariate analysis (solitary cases only)

Factors	Category	Hazard ratio (95% CI)	*P* value
Portal vein invasion	1: –	1	
	2: +	2.35 (1.31–4.20)	0.004
ASRI	1: < 20	1	
	2: ≥20	2.23 (1.19–4.18)	0.013
Serum albumin	1: ≥3.5	1	
	2: < 3.5	1.74 (1.05–2.88)	0.030

ASRI, α-foetoprotein–size ratio index; CI, confidence interval.

Table 5. Univariate analysis for clinical factors associated with advanced recurrence

Factors	Advanced recurrence, *n* = 22 (%)	Minor recurrence, *n* = 80 (%)	No recurrence, *n* = 63 (%)
Age			
< 65 years	13 (59)	59 (73.8)	35 (55.6)
≥65 years	9 (41)	21 (26.2)	28 (44.4)
Gender			
Male	19 (86.4)	62 (77.5)	46 (73)
Female	3 (13.6)	18 (22.5)	17 (27)
HBV			
Positive	5 (22.7)	19 (23.8)	22 (34.9)
Negative	17 (77.3)	61 (76.2)	41 (65.1)
HCV			
Negative	5 (22.7)	23 (28.8)	28 (44.4)
Positive	17 (77.3)	57 (71.2)	35 (55.6)
Serum albumin			
≥3.5	11 (50)	49 (61.3)	41 (65.1)
< 3.5	11 (50)	31 (39.7)	22 (34.9)
Serum bilirubin			
< 1.5	21 (95.5)	67 (83.8)	56 (88.9)
≥1.5	1 (4.5)	13 (16.2)	7 (11.1)
AST levels			
< 50	17 (77.3)	40 (50)	42 (66.7)
≥50	5 (22.7)	40 (50)	21 (33.3)
Prothorombin time			
< 70	2 (9.1)	5 (6.3)	14 (22.2)
≥70	20 (90.9)	75 (93.7)	49 (77.8)
ICG R 15			
< 30	15 (68.2)	58 (72.5)	48 (76.2)
≥30	7 (31.8)	22 (27.5)	15 (23.8)
Platelet count			
$< 10^5$	7 (31.8)	28 (35)	30 (47.6)
$\geq 10^5$	15 (68.2)	52 (65)	33 (52.4)
AFP levels			
< 1000	17 (77.3)	73 (91.3)	61 (96.8)
≥1000	5 (22.7)*	7 (8.7)	2 (3.2)
ASRI			
< 20	15 (68.2)	70 (87.5)	60 (95.2)
≥20	7 (31.8)*	10 (12.5)	3 (4.8)
DCP levels			
< 100	18 (81.8)	68 (85)	54 (85.7)
≥100	4 (18.2)	12 (15)	9 (14.3)
Fibrosis stage			
F1, 2, 3	9 (41)	23 (28.8)	19 (31.7)
F4	13 (59)	57 (71.2)	41 (68.3)
Tumour size			
< 21	11 (50)	53 (66.3)	37 (58.7)
≥21	11 (50)	27 (33.7)	26 (41.3)
Tumour number			
Solitary	14 (63.6)	67 (83.8)	58 (92.1)
Multiple	8 (36.4)*	13 (16.2)	5 (7.9)
Vascularity			
Negative	1 (4.5)	3 (3.8)	8 (12.7)
Positive	22 (95.5)	77 (96.2)	55 (87.3)
Tumour differentiation			
Early, well, moderately	13 (59.1)	74 (92.5)	56 (88.9)
Poorly	9 (40.9)*	6 (7.5)	7 (11.1)
Eg			
Eg	21 (95.5)	63 (78.8)	54 (85.7)
Ig	1 (4.5)	17 (21.2)	9 (14.3)
Capsular formation			
Absence	6 (27.3)	33 (41.3)	22 (34.9)
Presence	16 (72.7)	47 (58.7)	41 (65.1)
Infiltration to capsular			
Absence	13 (59.1)	54 (67.9)	46 (73)
Presence	9 (40.9)	26 (32.1)	17 (27)
Septum formation			
Absence	16 (72.7)	61 (76.2)	46 (73)
Presence	6 (27.3)	19 (23.8)	17 (27)
Portal vein invasion			
Absence	17 (77.3)	65 (81.3)	54 (85.7)
Presence	5 (22.7)	15 (18.7)	6 (14.3)
Intrahepatic extent of tumour			
Absence	20 (90.9)	78 (97.5)	59 (98.3)
Presence	2 (9.1)	2 (2.5)	1 (1.7)

*Significantly higher than the other groups ($P < 0.05$).

AFP, α-foetoprotein; ASRI, α-foetoprotein–size ratio index; AST, aspartic transaminase; DCP, des-γ-carboxy prothorombin; Eg, expansive growth (well-demarcated border); HBV, hepatitis B virus; HCV, hepatitis C virus; ICG R15, indocyanine green retention test at 15 min; Ig, infiltrative growth (poorly demarcated border).

Table 6. Predictive factors of advanced recurrence after curative resection by multivariate analysis using the Cox model

Factors	Category	Hazard ratio (95% CI)	*P* value
Tumour number	1: solitary	1	
	2: multiple	5.65 (1.77–18.1)	0.003
ASRI	1: < 20	1	
	2: ≥20	4.04 (1.16–14.1)	0.028
Tumour differentiation	1: early, well, moderately	1	
	2: poorly	2.70 (1.51–4.82)	0.001

ASRI, α-foetoprotein–size ratio index; CI, confidence interval.

presence of extrahepatic metastasis (extrahepatic metastasis group). The multi/large nodular recurrence group had 17 cases (77.3%), the vascular invasion group had three (13.6%) and the

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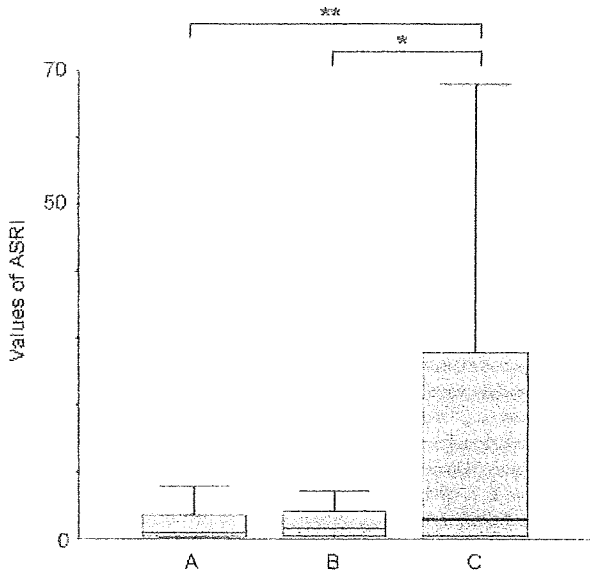

Fig. 1. Comparison with values of ASRI by patterns of recurrences. (A) No recurrence group, (B) minor recurrence group, (C) advanced recurrence group. $^{*}P=0.032$, $^{**}P=0.028$.

extrahepatic metastasis group had two (9.1%) in 22 cases of advanced recurrence. In particular, patients in the vascular invasion group had significantly higher pre-operative des-γ-carboxy prothorombin levels than those in the other two groups ($P=0.008$). Meanwhile, there was no significant difference of ASRI among the three groups.

Survival rate after curative resection by patterns of recurrences

Figure 2 shows the overall survival rates by patterns of recurrences. The overall survival rates of patients were 98.5, 93.6 and 91.8% for the first, third and fifth year in the no recurrence group; 98.8, 96.5 and 85.6% in the minor recurrence group; and 91.3, 64.5 and 35.1% in the advanced recurrence group respectively. The overall survival rates of the advanced recurrence group were significantly lower than those of the minor recurrence and the no recurrence groups (advanced recurrence vs. no recurrence: $P < 0.0001$, advanced recurrence vs. minor recurrence: $P=0.001$). Furthermore, the overall survival rates of the minor recurrence group were significantly lower than those of the no recurrence group ($P=0.009$). However, the overall survival rates of both the minor recurrence and the no recurrence groups were similar for the first 5 years after surgery.

Discussion

Our study identified the clinical, radiological and histological factors associated with advanced tumour recurrence and prognosis after curative resection of small HCC. Predictive factors of advanced recurrence were tumour number, ASRI and tumour differentiation. ASRI, which was made to reflect the malignant potential of HCC precisely, was easy to calculate and useful to predict the overall and advanced recurrence of HCC. Patients in the advanced recurrence group had a poorer prognosis than those in the minor recurrence and the no recurrence groups. On the other hand, patients in the minor recurrence group had a

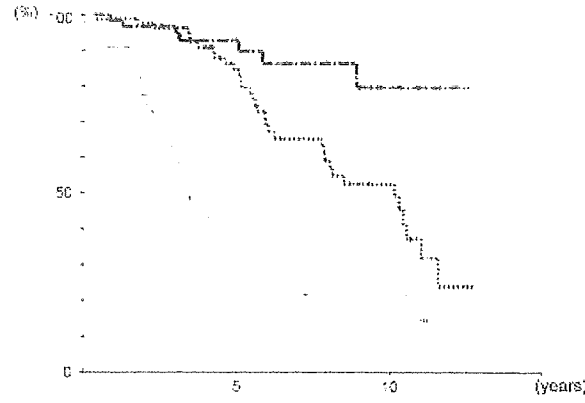

Fig. 2. Overall survival rates by patterns of recurrences; thick broken line: no recurrence group, dot line: minor recurrence group, solid line: advanced recurrence group.

prognosis similar to that of the no recurrence group for the first 5 years after resection.

Some predictors of survival and recurrence after resection were reported previously (21–24). These reports showed that the main predictors of recurrence were tumour size, tumour number, serum AFP levels, tumour differentiation, vascular invasion, etc. In the present study, we intended for patients with small HCC within 3 cm to pick up cases with high malignant potential. Therefore, tumour size was not associated with recurrence, but the other factors mentioned above were associated with recurrence as well as previous reports. However, we recently showed that ASRI was associated with both overall and advanced recurrence after resection. Small HCC with a high ASRI value may have a high malignant potential and may be likely to cause intra- or extrahepatic metastasis.

The high recurrence rate of HCC after curative resection and ablation is attributable to two principal characteristics: intrahepatic metastasis and de novo multicentric carcinogenesis. Some studies have shown that intrahepatic metastasis is an important mechanism of early recurrence after resection (13, 16, 24). In the present study, time to advanced recurrence was short: just 1 year. Furthermore, a previous study showed that tumour differentiation, which was a predictive factor of advanced recurrence in this study, was associated with intrahepatic metastasis (22). This is probably because potential metastasis depends on biological tumour factors, such as tumour differentiation. Considering these facts, a main mechanism of advanced recurrence is assumed intrahepatic metastasis. High AFP levels have been reported as a poor prognosis factor after resection of HCC (25, 26). On the other hand, it is assumed that AFP levels may increase in patients with acute or chronic active inflammation in background hepatocytes without HCC (27, 28). It is difficult to distinguish these mechanisms of AFP elevation. We created ASRI to evaluate the malignant potential of HCC by calculating AFP values per unit tumour diameter. Although it is impossible to distinguish neoplastic and inflammatory AFP elevation using this index, ASRI may mainly reflect neoplastic AFP elevation because ASRI is a predictive factor of advanced recurrence of HCC. In addition, Imamura *et al.* (24) reported that high AFP levels were associated with early recurrence within 2 years after resection, and this fact also supports our result.

α-foetoprotein levels usually tend to be higher in HBV-related HCC than those related to HCV, and this tendency has been reported by researchers in Japan, where HCV is

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predominant in HCC incidence (29). However, there was no significant difference in AFP levels between HBV- and HCV-related HCC in this study. We re-evaluated the predictive factors of recurrence after resection by stratifying this cohort into HBV- or HCV-related HCC. ASRI $\geq$ 20 was significantly associated with overall recurrence after resection in the HBV cohort, and this result was similar in HCV. Therefore, we consider ASRI as the useful index regardless of the viral aetiology, even in an HBV-endemic area.

Patients with advanced recurrence had a poor prognosis because of limitation and resistance of treatment. The overall survival rates were lower (35.1% per 5 years) in the advanced recurrence group than in the minor or the no recurrence group, in this study. On the other hand, patients with minor recurrence had a relatively good prognosis because it was possible to conduct reresection or percutaneous ablation therapy for recurrent tumour. Therefore, adjuvant therapy to prevent advanced recurrence after resection is needed. Although a number of studies of adjuvant therapy have been reported, none is effective for preventing intrahepatic metastasis after resection of HCC. Pre-/post-operative chemoembolization and chemotherapy had no benefit for tumour recurrence (30–32). Although a few authors including our hospital have reported that interferon is effective for preventing recurrence of HCC after resection, it is assumed that interferon itself suppresses de novo carcinogenesis (33–35). Recently, it was reported that sorafenib, which was a multikinase inhibitor, improved the overall survival rates in patients with advanced HCC (36). Sorafenib is expected to have the potential of effective adjuvant therapy to prevent tumour recurrence by intrahepatic metastasis, and a future report is awaited.

In conclusion, tumour number, ASRI and tumour differentiation were identified as risk factors for advanced recurrence of HCC. In particular, ASRI was easy to calculate and a useful index to predict advanced recurrence after curative resection of small HCC and to choose patients requiring adjuvant therapy after resection.

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Original Article

Effectiveness of combination therapy of splenectomy and long-term interferon in patients with hepatitis C virus-related cirrhosis and thrombocytopenia

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Aim: To elucidate the effectiveness of combination therapy of splenectomy and long-term interferon (IFN) on survival and hepatocarcinogenesis, we retrospectively analyzed 180 patients with hepatitis C virus (HCV)-related cirrhosis and thrombocytopenia.

Methods: Group A consisted of 121 patients who received neither splenectomy nor IFN therapy. Group B consisted of 11 patients who underwent splenectomy only. Group C consisted of 32 patients who underwent IFN therapy only. Group D consisted of 16 patients who received the combination therapy splenectomy followed by IFN therapy.

Results: The viral response in group D estimated at least 6 months after IFN therapy showed sustained viral response in four patients, biochemical response in one and no response in six. Multivariate analysis using time-dependent variables showed significant improvement of survival rate in patients on the combination therapy,but no effect on the appearance rate of hepatocarcinogenesis relative to the findings in group A.

Conclusions: In this study, the splenectomy did not directly improve the prognosis, but increased the ability for patients to undergo IFN. As a result, we considered that the combination therapy of splenectomy and long-term IFN significantly improved survival rate in patients with advanced HCV-related cirrhosis and thrombocytopenia.

Key words: cirrhosis, hypersplenism, interferon, splenectomy, thrombocytopenia

Abbreviations:
AFP, Alpha-fetoprotein; ALT, Alanine aminotransferase; AST, Aspartic aminotransferase; BR, biochemical response; CT, Computed tomography; HCC, Hepatocellular carcinoma; HCV, Hepatitis C virus; ICG R15, Indocyanine green retention rate at 15 min; IFN, Interferon; MELD score, Model for End-Stage Liver Disease score; NR, No response; PLT, platelet; SVR, Sustained virological response; TTT, Thymol turbidity test; US, Ultrasonography; ZTT, Zinc sulfate turbidity test.

INTRODUCTION

THE PRESENCE OF severe thrombocytopenia in patients with cirrhosis associated with hepatitis C viral (HCV) infection limits the use of interferon (IFN) therapy. The different treatment modalities for hepatocellular carcinoma (HCC), such as hepatic resection, radiofrequency ablation, or percutaneous ethanol injection, are also limited by low platelet (PLT) counts. In patients with compensated cirrhosis and low model for end-stage liver disease (MELD) score, liver transplantation is not warranted and the use of antiviral therapy to slow down the progression to liver failure is not recommended. In other words, such patients are too healthy for transplantation and too thrombocytopenic to treat with antiviral agents. Splenectomy has been suggested for the treatment of secondary hypersplenism and thrombocytopenia as a means to improve PLT count.[1]

If patients with HCV-related cirrhosis and thrombocytopenia could receive the benefits of splenectomy[2,3] and IFN therapy,[4,5] such therapy would clinically be very useful. The combination therapy of splenectomy and long-term IFN administration may improve survival rate and reduce the incidence of hepatocarcinogenesis.

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However, there are only a few reports that have examined the usefulness of this combination therapy in patients with advanced HCV-related cirrhosis and low PLT count.[6] In this study, we retrospectively analyzed 180 patients with compensated cirrhosis and thrombocytopenia who had received the combination therapy of splenectomy and long-term IFN to determine the effects of such treatment on the survival rate and incidence of HCC.

PATIENT AND METHODS

Study population

A TOTAL OF 180 Japanese patients with cirrhosis, hypersplenism and low PLT count ($\leq 80 \times 10^3/\mu L$) were examined between 1990 and 2006. Their initial sera were positive for antibodies to HCV (anti-HCV; second-generation anti-HCV kit; ELISA, Dainabot, Tokyo, Japan), positive HCV-RNA (Amplicor HCV monitor assay version 2.0; Roche Diagnostics, Tokyo, Japan), and negative for hepatitis B surface antigen (HBsAg; radioimmunoassay, Dainabot). Anti-HCV was assayed using stored frozen sera at -80°C. They were diagnosed with liver cirrhosis between 1990 and 2006 at Toranomon Hospital, Tokyo, Japan. In addition to liver biopsy and/or peritoneoscopy, liver cirrhosis was also diagnosed utilizing clinical findings (e.g. presence of esophageal varices), and with computed tomographic (CT) or ultrasonographic (US) findings. The following protocol was applied in our hospital until 2000: Patients with a platelet count of less than $50 \times 10^3/\mu L$ are eligible for HCC surgery (such as hepatic resection, radiofrequency ablation, or percutaneous ethanol injection) provided they receive platelet transfusion. The decision to pursue splenectomy was individualized and based on the presence thrombocytopenia and/or intractable gastric varices, and discussed with the patients.

We retrospectively analyzed the effect of splenectomy on cirrhotic patients with low PLT count ($\leq 80 \times 10^3/\mu L$). Of the total 180 patients, 121 (67.2%) patients received neither antiviral therapy nor splenectomy (group A). Thirty-two (17.8%) patients received only IFN therapy (group C). The remaining 27 (15.0%) patients underwent splenectomy (11 patients underwent only splenectomy [group B] and 16 received IFN therapy after splenectomy [group D]). Splenectomy was performed for the following reasons; (i) low PLT count in 20 patients (six [54.5%] of group B and 14 [8.5%] of group D), (ii) low PLT count and part of treatment of gastric varices in three (one [9.0%] of group B and two [12.5%] of group D), and (iii) low PLT count and refractory esophageal varices in four (four [36.4%] of group B). None of the patients required emergency splenectomy (e.g. bleeding gastric varices or other bleeding complications related to low platelet count). Our institution does not require informed consent for retrospective analysis.

Patients background and laboratory data

Table 1 summarizes the profiles and patients of groups A, B, C and D at the time of diagnosis of liver cirrhosis. Indocyanine green test was conducted in 91.2% of the patients. Patients of group D had significantly lower PLT count ($P = 0.01$) and AST ($P = 0.01$) than patients in others groups. The proportion of group A patients who regularly consumed alcohol at ≥ 80 g/day was significantly higher than other groups. Patients of group C had significantly lower TTT ($P = 0.08$) than others.

Splenectomy

Splenectomy was performed through midline or left subcostal incision depending on body habitus and previous incisions. For group B, five patients underwent splenectomy and six underwent Hassab's operation.[7] In group D, 13 patients underwent splenectomy and three underwent Hassab's operation.

IFN treatment

Thirty-two patients received IFN therapy (group C). In group C, 21 patients received 3 million units of IFN-α (natural or recombinant) intramuscularly three times per week to maintain a low alanine aminotransferase (ALT), 11 patients received 6 million units of IFN-α to eradicate HCV. Patients of group C received IFN therapy for a median period of 0.5 years (range, 0.0–9.7 years).

Sisteen patients received the combination therapy (group D). Of these, 12 (75%) patients underwent splenectomy for the purpose of induction of antiviral therapy with IFN. The other patients (25%) had undergone splenectomy pre dating this study. In group D, 11 patients (Cases 1–4, 8, 10–13, 15–16) received 3 million units of IFN-α (natural or recombinant) intramuscularly three times per week to maintain a low ALT, 3 patients (Cases 6, 7, and 9) received 6 million units of IFN-α to eradicate HCV. For the other two patients; one (Case 5) received pegylated IFNα2b (50 μg) monotherapy and the other patient (Case 14) received pegylated IFNα2b (50 μg) plus ribavirin (400 mg) combination therapy to maintain low ALT (Fig. 1). Patients of group D received IFN therapy for a median period of 1.4 years (range, 0.2–12.4 years).

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Table 1 Patient profiles and laboratory data at the time of diagnosis of cirrhosis

	Group A (Neither splenectomy nor IFN)	Group B (splenectomy)	Group C (IFN)	Group D (splenectomy + IFN)	*P**
Demography					
No. patients	121	11	32	16	
Sex (M/F)	64/57	6/5	13/19	13/3	0.07
Age (years)†	61 (32–82)	61 (42–66)	59 (36–72)	52 (36–60)	0.41
Alcohol intake of 80 g/day or more	29	0	10	0	0.03
Diabetes mellitus	12	1	4	2	0.96
Laboratory data†					
Platelet count ($\times 10^3/\mu L$)	61 (17–80)	64 (42–75)	66 (25–80)	44 (27–78)	0.01
Prothrombin activity (%)	73 (50–101)	79 (58–94)	80 (66–100)	74 (47–100)	0.88
Albumin (g/dL)	3.5 (1.7–4.8)	3.5 (2.0–4.3)	3.4 (2.5–4.1)	3.3 (2.7–4.5)	0.64
ZTT (Kunkel)	12.3 (0.7–23.3)	10.3 (3.3–18.2)	10.8 (4.4–21.0)	12.0 (6.1–17.1)	0.29
TTT (Kunkel)	14.1 (0.4–37.2)	12.0 (4.4–16.9)	7.8 (1.2–34.0)	12.7 (2.7–34.1)	0.08
Bilirubin (mg/dL)	1.5 (0.4–7.7)	1.2 (0.7–5.3)	1.1 (0.6–2.7)	1.2 (0.8–4.4)	0.03
AST (IU/L)	64 (21–652)	83 (31–157)	75 (28–216)	60 (30–154)	0.17
ALT (IU/L)	53 (11–239)	72 (24–191)	71 (18–298)	46 (14–182)	0.01
ICG R15 (%)	38 (12–96)	41 (15–64)	32 (6–62)	32 (8–53)	0.44
Alpha-fetoprotein (ng/mL)	23 (2–909)	40 (3.9–165)	29 (5–631)	11 (4–190)	0.28

ALT, alanine aminotransferase; AST, aspartic aminotransferase; ICG R15, indocyanine green retention rate at 15 min; TTT, thymol tubidity test; ZTT, zincsulfate tubidity test.
*Kruskal-Wallis test or χ^2-test. †Expressed by median (min, max).

The effect of IFN therapy was classified according to elimination of HCV-RNA and ALT value 6 months after the end of treatment. Sustained virological response (SVR) was defined as persistent disappearance of HCV RNA after therapy, biochemical response (BR) as normal ALT values without elimination of HCV RNA for at least 6 months after therapy, and no response (NR) as persistently elevated or transiently normalized ALT levels without loss of HCV RNA.

Follow up of patients

Patients were followed up on a monthly basis after the diagnosis of cirrhosis by monitoring hematologic, biochemical, and virologic data. Imaging studies were conducted three or more times per year in the majority of patients by using computerized tomography (CT) or ultrasonography (US). Angiography was performed only when HCC was highly suspected based on CT or US. When angiography detected a typical hypervascular nodule, it was considered a specific finding for HCC in these follow-up patients, and histological confirmation was usually not required in the majority of patients. If the angiographic study did not show any hypervascular staining in a small hepatic nodule, a fine needle biopsy was performed. In this cohort, 18 (12.2%) patients were lost to follow up [14 patients (11.6%) from group A, two patients (18.2%) from group B, one patient (3.1%) from group C and two patients (12.5%) from group D]. The date of the last follow-up in this study was 31 March 2007, and the median observation period of studied patients was 5.9 years (range, 0.1–19.6 years).

Statistical analysis

Non-parametric procedures were used for the analysis of background characteristics of the patients, including Kruskal–Wallis and χ^2 test. Changes in laboratory tests values after splenectomy were evaluated by using Wilcoxon signed-rank test. Survival rate was calculated from the period between diagnosis of liver cirrhosis and death in each group, by using the Kaplan–Meier method.[8] HCC appearance rate was calculated from the period between diagnosis of liver cirrhosis and appearance of HCC in each group, by again using the Kaplan–Meier method. Differences in slopes of survival and carcinogenic curves were evaluated by log-rank test. The median waiting period between diagnosis of cirrhosis and splenectomy was 1.6 months (range, 0.0–199.5 months) for groups B and C. To compensate for wait-time bias in the splenectomy groups, curves of survival and HCC appearance were also drawn from the time of diagnosis

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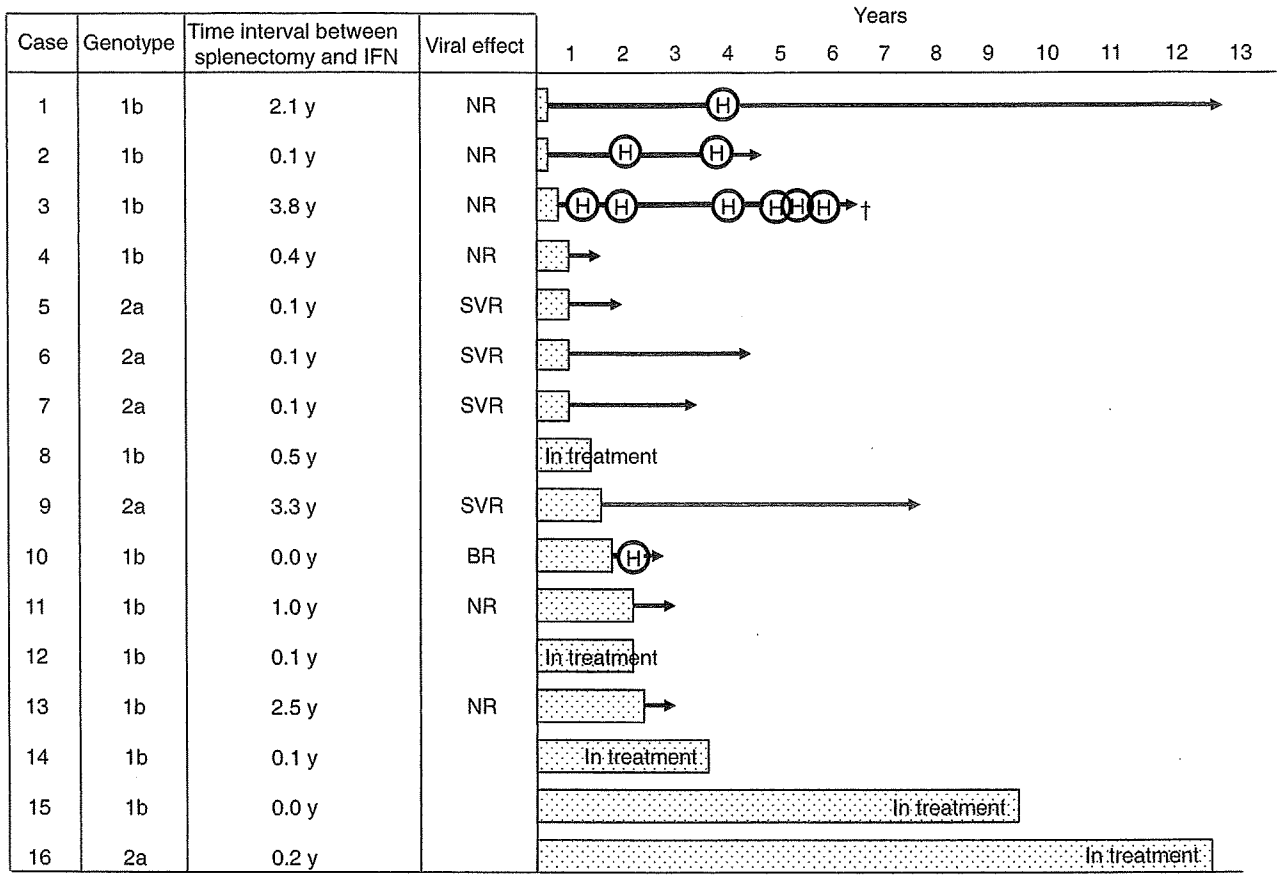

Figure 1 Individual patients who underwent splenectomy followed by long-term IFN therapy (group D). Hepatocellular carcinoma (HCC) developed in five of 16 patients. The dotted bars and arrows represent IFN therapy and follow-up period. H, appearance of HCC; SVR, sustained virological response; BR, biochemical response; NR, no response; †, death.

of cirrhosis in the groups. Independent factors associated with survival and HCC appearance were studied by using time-dependent Cox regression analysis.[9] The following 14 variables were analyzed for potential covariates for survival and liver carcinogenesis at the time of the diagnosis of cirrhosis: age, sex, habitual alcohol intake (80 g/day or more), association of diabetes, albumin, zinc sulfate turbidity test (ZTT), thymol turbidity test (TTT), bilirubin, aspartic aminotransferase (AST), ALT, PLT count, prothrombin activity, indocyanine green retention rate at 15 min (ICG R15), and alpha-fetoprotein (AFP). In addition to these variables, an interaction term of "waiting time" from the diagnosis of liver cirrhosis to splenectomy was introduced in the analysis as a time-dependent covariate. Several variables were transformed into categorical data consisting of two or three simple ordinal numbers in order to estimate the hazard ratio. All factors found to be at least marginally associated with survival and liver carcinogenesis ($P < 0.10$) were entered into multivariate Cox proportional hazard model. A P-value of less than 0.05 was considered to be significant. Statistical analyses were performed using the SPSS software (SPSS, Chicago, IL, USA).

RESULTS

Effects and complications of splenectomy

THE SPLENECTOMY GROUP consisted of 11 patients with Child–Pugh Class A (group B = 2, group D = 8), 15 with Child–Pugh Class B (group B = 8, group D = 7) and 1 with Class C (group D = 1) at operation. The median weight of the removed spleen was 430 g (range, 190–1600 g). Leukocyte count, PLT count and total bilirubin improved in most patients after sple-

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nectomy. Leukocyte count increased about 1.6 times at 6 months after splenectomy [before splenectomy, median = 3200/mm^3 (range 1800–5600); after splenectomy, 5200 (3700–9000); $P < 0.001$). PLT count increased about 2.3 times at 6 months after splenectomy [before splenectomy, median = $47 \times 10^3/\mu L$ (range, $26–77 \times 10^3$); after splenectomy, 110×10^3 ($79–275 \times 10^3$); $P < 0.001$). Total bilirubin decreased about 0.6 times at 6 months after splenectomy [before splenectomy, median = 1.2 mg/dL (range, 0.6–4.4); after splenectomy, 0.7 (0.4–1.8); $P = 0.001$). Leukocyte and PLT counts reached peak levels within a month after splenectomy and were almost stabilized at six months.

Postoperative complications following splenectomy developed in three patients; hemoperitoneum ($n = 1$), portal vein thrombosis ($n = 1$) and secondary thrombocytopenia ($n = 1$). Some patients received prophylactic anticoagulation to protect against portal vein thrombosis after splenectomy. One patient with hemoperitoneum died due to multiple organ failure, while the other patients recovered with medical treatment.

Complications of splenectomy plus IFN combination therapy

Figure 1 shows patients that underwent combination therapy (group D). During the observation period, one patient (Case 3) of group D died of liver failure caused by progression of HCC. The causes of death in three other patients were not deemed to be complications related to the combination therapy. None of the patients of group D developed serious complications (e.g. portal vein thrombosis, post-operative hemorrhage, pneumonia, sepsis) from the splenectomy. Postoperatively, none of the patients showed worsening of liver biochemical test results or developed decompensated liver disease with ascites, encephalopathy, jaundice or variceal bleeding. There were also no deaths in the immediate postoperative period. Three patients (18.8%) of group D discontinued IFN therapy for the following reasons; severe thrombocytopenia (Case 1), NSAID-induced liver injury (Case 2) and peripheral neuropathy (Case 13). In contrast, eight patients (25.8%) of group C discontinued IFN therapy. Three (37.5%) of them discontinued IFN therapy due to severe thrombocytopenia. When frequency of discontinued IFN therapy was compared with group C and D, there was no significant difference ($P = 0.73$). However, there were cases, eight in group C but 0 in group D, who required a reduction in IFN dosages during treatment as compared with the beginning of treatment ($P = 0.03$). The splenectomy could have increased the ability for patients to undergo IFN.

Effect of IFN therapy after splenectomy

Eleven of 16 (68.8%) patients of group D had HCV genotype 1b and five (31.3%) had HCV genotype 2a (Fig. 1). The viral response was determined at least 6 months after IFN therapy; SVR was noted in four (36.4%) patients, BR in one (9.1%) and NR in six (54.5%). Three patients continue to receive IFN therapy at present. In this study, patients with SVR were all male and had genotype 2a. One of the patients with SVR received pegylated-IFNα–2b (Case 5, Fig. 1), while other patients received IFNα2b. Meanwhile, 18 of 32 (56.3%) patients of group C had HCV genotype 1b, 12 (37.5%) had HCV genotype 2a and two (6.3%) had HCV genotype 2b. Group C had more patients with low HCV-RNA (< 100 000 IU/mL) than group D (12 [37.5%] of group C and three [18.8%] of group D, $P = 0.09$). In group C, SVR was noted in 7(21.9%) patients, BR in six (18.8%) and NR in 17 (53.1%). Two patients continue to receive IFN therapy at present.

SVR were not significantly different between group C and D ($P = 0.43$). This result might be a reason that group D had more patients with HCV genotype 1 and higher HCV-RNA than group C.

Rate of hepatocarcinogenesis

During the follow-up period of up to 17 years (median observation period of 5.9 years), HCC developed in 65 patients (36.1%): 40 (33.1%) in group A, five (45.5%) in group B, 16 (50.0%) in group C and four (25.0%) in group D. HCC appearance rates at the end of the third year were 19.9, 20.0, 25.0 and 6.3% in group A, B, C and D, 28.5, 57.3, 34.5 and 14.1% at the end of the fifth year, and 48.2, 78.7, 43.8 and 39.8% at the end of tenth year, respectively (Fig. 2). There was no significant difference in the rate of HCC appearance among the four groups (log-rank test, $P = 0.42$). In particular, the HCC appearance rate in group D was not significantly different compared with group A (log-rank test, $P = 0.50$).

In addition, the rate of carcinogenesis correlated inversely with the duration of IFN administration (Fig. 1). For group D, 9 of 14 patients were treated with IFN for ≥ 12 months. The carcinogenic rate at the end of the 5th year in the remaining patients of the same group who were treated with IFN for < 12 months (20.0%) was higher than in those treated for ≥ 12 months (9.1%). Multivariate analysis showed that the hazard ratio of carcinogenesis for patients treated with IFN for

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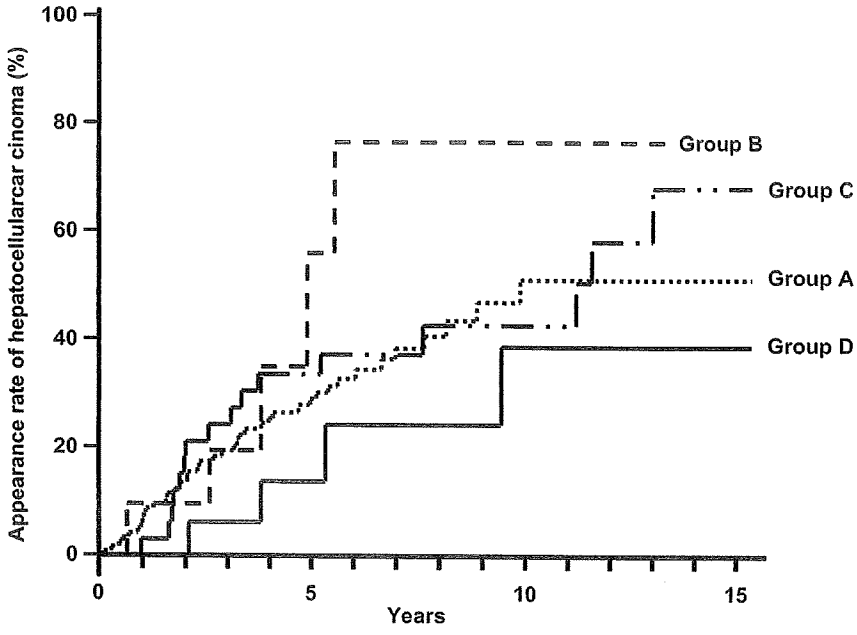

Figure 2 Crude hepatocellular carcinogenesis (HCC) curves in patients of groups A, B, C and D. There was no significant difference in the HCC appearance rate among the four groups (log-rank test, $P = 0.42$).

≥ 12 months was 0.022 after adjustments for significant covariates, but was not significantly different ($P = 0.43$).

We also assessed the effects of splenectomy and long-term IFN therapy on hepatocarcinogenesis by comparing patients of group D (splenectomy + IFN administration for ≥ 12 months) with those of group A. The combination therapy reduced the hazard ratio to 0.03 (multivariate analysis with adjustments for significant covariates), though it was significant ($P = 0.83$). We also assessed compared patients of groups C and B (splenectomy alone). Administration of IFN for ≥ 12 months reduced the hazard ratio to 0.03 (multivariate analysis after adjustments for significant covariates), but was not significant ($P = 0.83$). These results suggest that the combination of splenectomy plus long-term IFN decreased the likelihood of hepatocarcinogenesis.

Effect of splenectomy and IFN combination therapy on survival

During the observation period, one of the 16 patients of group D (Case 3) died (Fig. 1). The survival rates for groups A, B, C and D were 84.2, 90.9, 87.5 and 100% at the end of the third year, 72.0, 90.9, 87.5 and 100% at the fifth year, 41.4, 36.4, 83.3 and 83.3% at the tenth year, respectively (Fig. 3). The survival rate for patients of group D was the highest compared with the other groups (log-rank test, $P = 0.002$). We also compared the effect of combination therapy on the survival rate of patients of group A and group D. The survival rate of group D was significantly higher than of group A (log-rank test, $P = 0.004$). We also compared the effect of combination therapy on the survival rate of patients of group C and group D. The survival rate of group D was not significantly different compared with group C (log-rank test, $P = 0.29$). The combination therapy significantly improved the hazard ratio of survival to 9.69 ($P = 0.028$, multivariate analysis with adjustments for significant covariates, Table 2). These results suggest that the splenectomy simply increased the ability for patients to undergo IFN and may not directly improve patient survival.

DISCUSSION

CHRONIC HEPATITIS C virus (HCV) will continue to cause significant morbidity and mortality through to at least 2015.[10] HCV infection remains a common cause of chronic liver disease and is an increasing indication for liver transplantation. Thrombocytopenia (platelet counts $< 150 \times 10^3/\mu L$) is a common complication in patients with chronic liver disease (CLD), and is reported in as many as 76% of cirrhotic patients.[11]The ability to increase platelet levels could significantly reduce the need for platelet transfusions and facilitate the use of IFN-based antiviral therapy and other medically indicated treatments in patients with liver disease. Current treatment options for severe

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