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)

Table 5. Continued

Factors	Advanced recurrence, *n* = 22 (%)	Minor recurrence, *n* = 80 (%)	No recurrence, *n* = 63 (%)
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

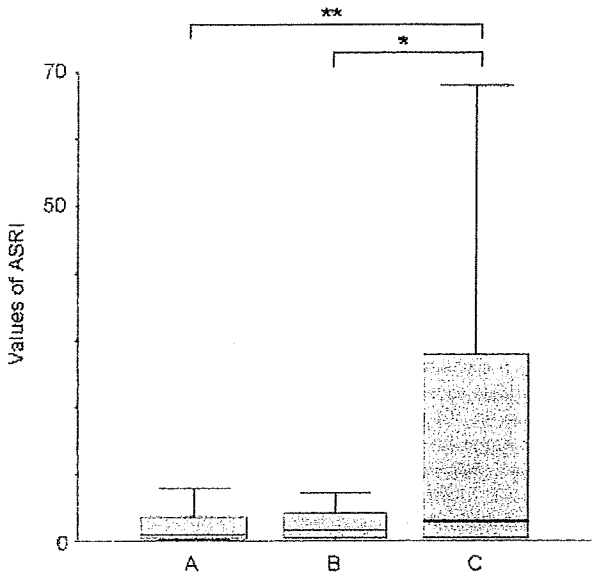

Fig. 1. Comparison with values of ASRI by patterns of recurrences. (A) No recurrence group, (B) minor recurrence group, (C) advanced recurrence group. *P=0.032, **P=0.028.

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

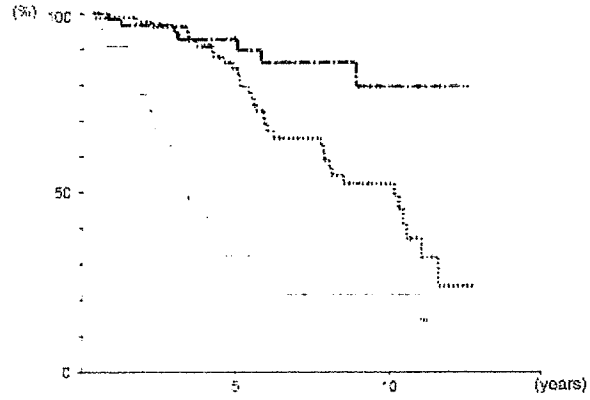

Fig. 2. Overall survival rates by patterns of recurrences; thick broken line: no recurrence group, dot line: minor recurrence group, solid line: advanced recurrence group.

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

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≥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).

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

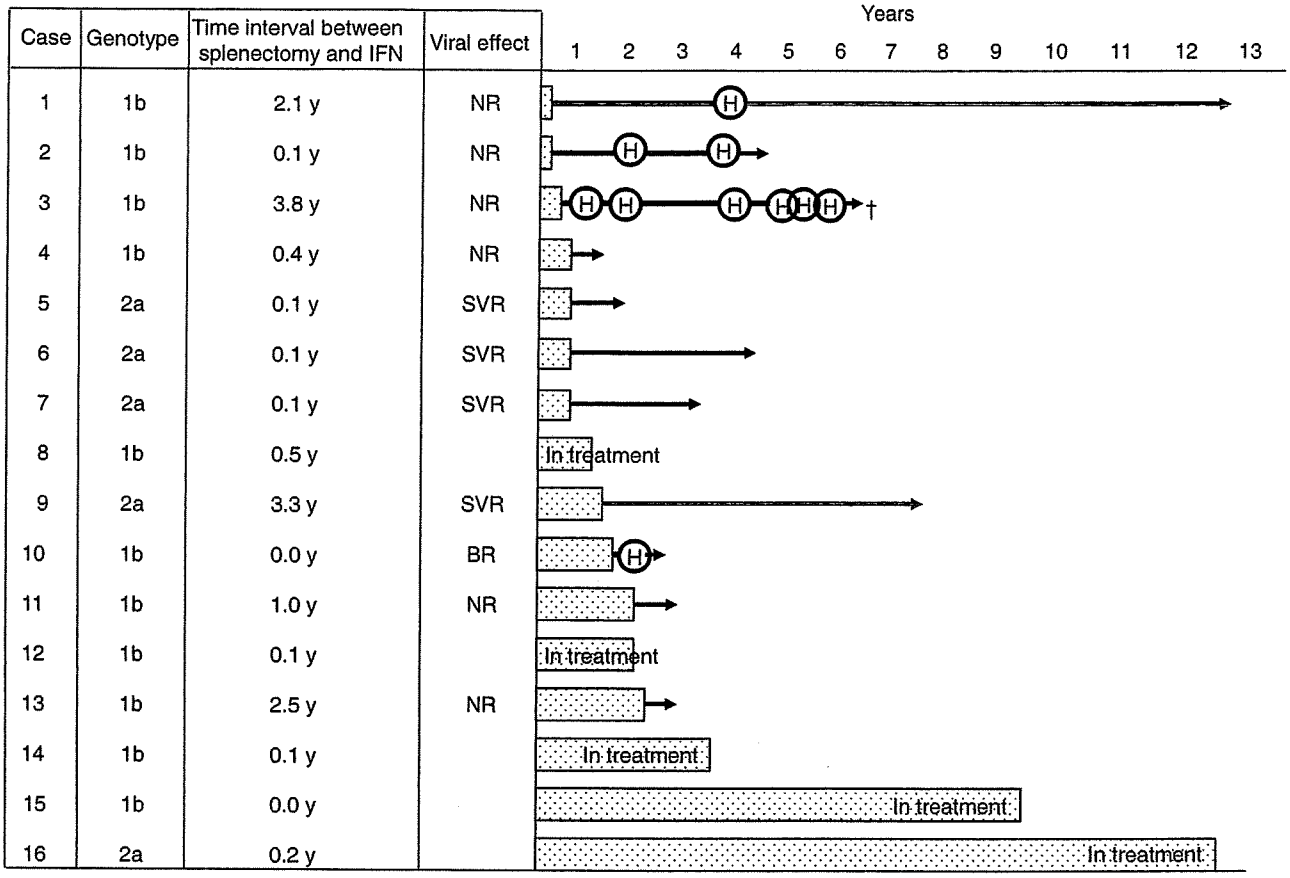

Figure 1 Individual patients who underwent splenectomy followed by long-term IFN therapy (group D). Hepatocellular carcinoma (HCC) developed in five of 16 patients. The dotted bars and arrows represent IFN therapy and follow-up period. H, appearance of HCC; SVR, sustained virological response; BR, biochemical 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-

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

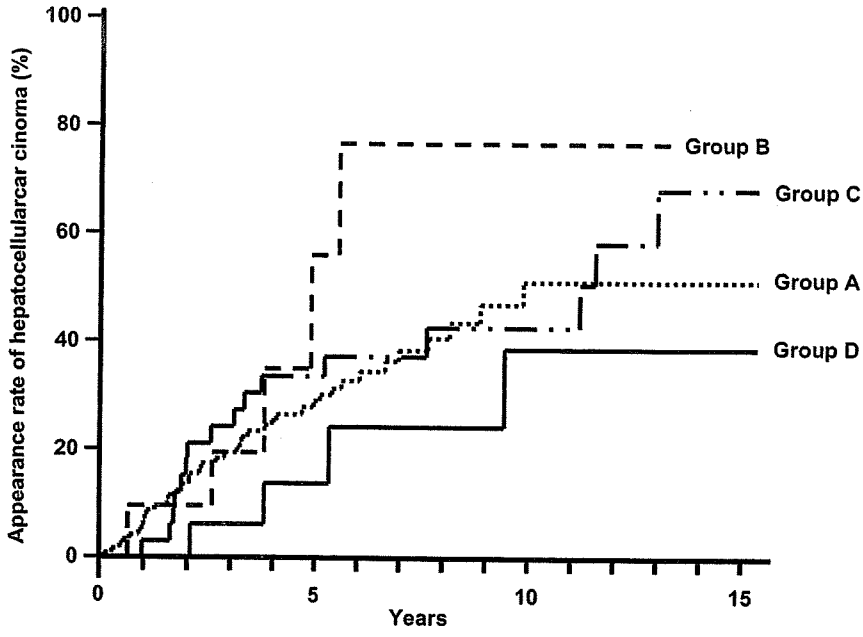

Figure 2 Crude hepatocellular carcinogenesis (HCC) curves in patients of groups A, B, C and D. There was no significant difference in the HCC appearance rate among the four groups (log-rank test, $P = 0.42$).

≥ 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

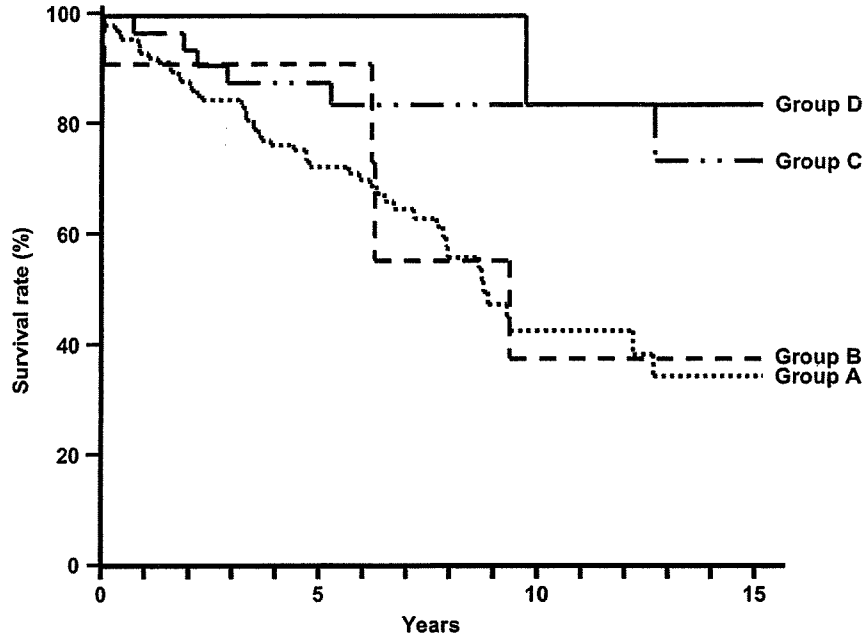

Figure 3 Survival rates for patients of groups A, B, C and D. The survival rate was significantly different for group A, B, C and D (log-rank test, $P = 0.002$). The survival rate of patients of group D was significantly higher than that of group A (log-rank test, $P = 0.004$).

thrombocytopenia include platelet transfusion, splenic artery embolization and splenectomy. We studied the usefulness of the combination therapy of splenectomy and long-term IFN in patients with advanced HCV-related cirrhosis and thrombocytopenia.

With regard to the usefulness of splenectomy, some studies reported that splenectomy improved PLT counts in cirrhotic patients with thrombocytopenia.[2,3] Furthermore, Shimada *et al.*[12] reported that splenectomy resulted in significant falls in ammonia levels and rises in serum albumin. Thus, there is evidence that splenectomy is beneficial and results in recovery of liver function by improving of blood supply to the liver.[6,13] In the present study, at 6 months after splenectomy, leukocyte count increased 1.6 times, PLT count increased 2.3 times, and total bilirubin decreased nearly 0.6 times, relative to prior the procedure. Furthermore, liver function test results also improved in most patients with splenectomy.

Table 2 Significance of combined therapy of survival rate in patients of advanced hepatitis C virus-related cirrhosis with low platelet count (time-dependent proportional hazard model)

Factors	Category	Hazard ratio (95% CI)	*P*
Combined therapy (splenectomy + IFN)	1: no 2: yes	1 9.69 (1.28–76.9)	0.028

IFN, interferon therapy.

With regard to the value of IFN therapy after splenectomy, Hayashi *et al.*[6] reported that splenectomy in patients with HCV cirrhosis can be done safely to allow application of antiviral treatment and potentially avoid transplantation.[6] In this study, only three of 16 (18.8%) patients discontinued IFN therapy after splenectomy. Among the three patients, IFN therapy was discontinued because of thrombocytopenia in only one (6.3%) patient. On the other hand, 13 (81.3%) of the 16 patients on combination therapy were able to complete the full course of IFN therapy, continue IFN therapy or stopped therapy due to NR. Thus, it may be said that IFN therapy is safe in most patients with advanced HCV-related cirrhosis and thrombocytopenia. Furthermore, the present results indicate that splenectomy is an effective method in patients with chronic HCV infection and hypersplenism to increase peripheral leukocyte and platelet counts so that subsequent IFN therapy can be better tolerated. In this study, regarding the reduction of IFN dosages during treatment when comparing group C and D, group D did not have any cases who a reduction in IFN dosages was necessitated by thrombocytopenia ($P = 0.03$). Hayashi *et al.*[6] reported that five of their seven patients underwent splenectomy and then completed a full course of pegylated IFN and ribavirin

treatment or stopped therapy due to NR, and that none of their patients required dose reductions or treatment discontinuation due to thrombocytopenia. In the present study, the viral response to IFN therapy was SVR in four (36.4%) patients, BR in one (9.1%) and NR in six (54.5%). SVR was 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. All patients with SVR of group D had genotype 2, suggesting that SVR seems to be achievable by combination therapy in patients with HCV-related cirrhosis with genotype 2 and thrombocytopenia.

We also analyzed the effect of the combination therapy on hepatocarcinogenesis in patients with advanced HCV-related cirrhosis and low PLT count. Chen *et al.*[14] reported that the 5-year tumor-free survival rate was significantly higher after hepatectomy and splenectomy than after hepatectomy alone (37 vs. 27.3%, respectively, $P = 0.003$). In contrast, Yao *et al.*[15] reported that splenectomy in early stage of tumor inoculation stimulated tumor growth and metastasis in their rat model of HCC.[15] In this study, the HCC appearance rate in patients who underwent splenectomy alone (group B) was not significantly different from that of the control (log-rank test, $P = 0.52$). In addition, the HCC appearance rate in patients who received the combination therapy was also not significantly different from the control (log-rank test, $P = 0.50$). We previously reported that long-term IFN therapy for 12 months or longer reduced the rate of hepatocarcinogenesis in patients with liver cirrhosis caused by HCV.[5] Multivariate analysis of long-term follow-up showed that the combination therapy, including IFN administration for ≥ 12 months, decreased the hazard ratio of hepatocarcinogenesis to 0.03, though this was not significant ($P = 0.83$). The reason for the lack of significance might be the small population sample of this study. Yoshida *et al.*[16] reported that IFN therapy significantly reduced the risk for HCC, especially among virologic and biochemical responders. That the combination therapy decreased the hazard ratio of hepatocarcinogenesis to 0.03 suggests the ability of long-term IFN to inhibit HCC, especially among non-responders.

We also examined the effects of the combination therapy on survival. In this study, multivariate analysis using time-dependent variables showed significant improvement of survival in patients who received the combination therapy (group D) compared with the control group (group A) (hazard ratio 3.40, $P = 0.017$; 95% CI 1.24–9.35). This may be considered the crucial finding of this study. In splenectomy, Morimasa *et al.*[17] reported no difference in survival rate between splenectomy and endoscopic injection sclerotherapy (EIS) for esophageal varices. Similarly, the survival rate in the splenectomy group in this study (group B) was not significantly different from the control ($P = 0.88$). Furthermore, the survival rate of group D was not significantly different compared with group C (log-rank test, $P = 0.29$). These results suggest that the splenectomy increased the ability for patients to undergo IFN and that the combination therapy of splenectomy and long-term IFN significantly improved survival rate in patients with advanced HCV-related cirrhosis and thrombocytopenia. The likely mechanism of action of the combination therapy is first improvement of leucopenia and thrombocytopenia following splenectomy, which allowed administration of IFN, and then IFN produced remission of liver fibrosis, control of necroinflammatory process, and induced suppression of the HCC growth process, consequently leading to improvement of survival rate. Moreno and Muriel[18] reported that IFN resulted in remission of liver fibrosis, and that control of the necroinflammatory process can therefore induce suppression of the HCC growth process. Our results also suggested that patients with NR may need to continue the combination therapy with long-term IFN therapy.

"Pegylated IFN plus ribavirin" and "eltrombopag" are promising drugs and can be potentially used in combination therapy. Recent multicenter trials have demonstrated the superiority of pegylated IFN plus ribavirin compared to pegylated IFN alone or non-pegylated combination therapy.[19,20] In addition, several promising novel agents that stimulate TPO and increase PLT count, such as the oral platelet growth factor eltrombopag, are currently in development for the prevention and/or treatment of thrombocytopenia.[21] Eltrombopag may be a substitute for splenectomy or PSE. Thus, combination therapy of pegylated IFN plus ribavirin after splenectomy or eltrombopag may improve survival rate and reduce the rate of hepatocarcinogenesis.

Our study had certain limitations. In particular, in this study, four (25%) of the patients who underwent combination therapy had a history of splenectomy. A randomized control study with a larger number of cases should be conducted to confirm the effectiveness of this therapy.

In conclusion, the combination therapy of splenectomy and long-term IFN decreased the rate of hepatocarcinogenesis and significantly improved the survival rate in patients with advanced HCV-related cirrhosis and low PLT count.

ACKNOWLEDGMENTS

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GASTROENTEROLOGY

Efficacy of entecavir treatment for lamivudine-resistant hepatitis B over 3 years: Histological improvement or entecavir resistance?

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Key words
entecavir, chronic hepatitis, hepatitis B virus, lamivudine, YMDD mutants.

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Abstract

Background and Aims: Long-term lamivudine therapy is required for patients with chronic hepatitis B, because hepatitis reappears frequently after it has withdrawn. However, hepatitis B virus (HBV) mutants resistant to lamivudine emerge frequently accompanied by breakthrough hepatitis.

Methods: Effects of entecavir were evaluated in 19 patients who had developed breakthrough hepatitis during lamivudine therapy for longer than 5 years. This study is a subgroup analysis of a previously reported study. Entecavir, in either 0.5 or 1.0 mg/day doses, was given to 10 and nine patients for 52 weeks, respectively, and then all received 1.0 mg/day entecavir for an additional 68–92 weeks.

Results: There were no differences in biochemical and virological responses in the two groups of patients with respect to the two different initial doses of entecavir. Serum levels of alanine aminotransferase were normalized in 17 (90%) patients, and hepatitis B e antigen (HBeAg) disappeared from the serum in two (14%) of the 14 patients who were HBeAg-positive before. Furthermore, a decrease in histological activity index score greater than 2 points was achieved in nine of the 11 (82%) patients in whom annual liver biopsies were performed during 3 years while they received entecavir. HBV mutants resistant to entecavir emerged in five of the 19 (26%) patients, and hepatitis flare occurred in two of them (40%).

Conclusion: Entecavir in the long term would be useful for histological improvement of breakthrough hepatitis induced by lamivudine-resistant HBV mutants in patients with chronic hepatitis B. However, the relatively high rate of entecavir resistance is a concern, and other strategies need to be considered when available.

Introduction

Worldwide, an estimated 400 million people are infected with hepatitis B virus (HBV) persistently, and some of them develop fatal liver disease, such as decompensated cirrhosis and hepatocellular carcinoma.[1] In 1995, lamivudine was introduced to the treatment of chronic hepatitis B for which interferon (IFN) had previously been the only option.[2,3] Although lamivudine is efficient for treatment of chronic hepatitis B, drug-resistant HBV variants with mutations in the tyrosine–methionine–aspartate–aspartate (YMDD) motif occur increasingly more frequently with treatment duration, to higher than 60% within 5 years.[4–7] Furthermore, these YMDD mutants are often accompanied by breakthrough hepatitis, and it is difficult to obtain disease control with lamivudine.

Subsequently, adefovir dipivoxil has been approved for treatment of chronic hepatitis B,[8,9] and more recently entecavir.[10–12] Entecavir is superior to lamivudine as the first-line treatment, and both adefovir add-on lamivudine and entecavir as switch therapy have also been employed for treatment of breakthrough.[13,14]

The present study represent a subgroup analysis of our previously reported multicenter randomized controlled trial.[12] From a single center, biological and virological responses to entecavir were examined among 19 patients who had developed hepatitis breakthrough during long-term lamivudine therapy, with particular focus on histological responses to entecavir over 3 years and the rate of development of entecavir resistance. Because patients had been randomized to both the low (0.5 mg) and higher (1.0 mg) doses of entecavir, we were also able to compare results between these two different doses.

Methods

Patients

During 10 years from November 1995 to December 2004, 704 patients with chronic hepatitis B received 100 mg lamivudine/day and were followed for more than 5 years in the Department of Hepatology of Toranomon Hospital in metropolitan Tokyo. Lamivudine-resistant YMDD mutants developed in 274 (39%) of the patients, accompanied by breakthrough hepatitis in 176 (64% of those with mutants). Medication was changed so they received the other antivirals. The present study is a subgroup analysis of our previously reported multicenter randomized controlled trial.[12] After entecavir became available, 19 of them were switched to it and the treatment was continued for up to 3 years. None of them were infected with hepatitis C virus (HCV) or HIV type 1, or had autoimmune hepatitis. They were followed for liver function tests and serum markers of HBV infection monthly. At the start of entecavir therapy, chronic hepatitis was diagnosed in them all by liver biopsies performed under laparoscopy and/or ultrasonic imaging; cirrhosis was detected in no patients. Liver biopsies were performed annually for 3 years on 12 of the 19 (63%) patients, for evaluating the efficacy of long-term entecavir in improving histology of the liver. The study design conformed to the 1975 Declaration of Helsinki, and was approved by the ethics committee of the institution. All patients gave their informed consent to participate in this study.

Markers of HBV infection

Hepatitis B surface antigen (HBsAg) and the corresponding antibody (anti-HBs) were determined by hemagglutination (MyCell; Institute of Immunology, Tokyo, Japan), and hepatitis e antigen (HBeAg) by enzyme-linked immunosorbent assay (ELISA) (F-HBe; Sysmex, Kobe, Japan). HBV-DNA was determined by reverse transcription polymerase chain reaction (RT-PCR) with commercial kits (Amplicor, Tokyo, Japan; Roche, Tokyo, Japan), and the result was expressed in log genome equivalents (LGE)/mm with the cut-off value of 2.6 LGE/mL over a dynamic range of 2.6–7.6 LGE/mL. The six major genotypes (A–F) were determined serologically by ELISA (HBV Fenotype EIA; Institute of Immunology). The method employs the combination of epitopes on preS2-region products that is specific for each genotype.[15,16]

Analyses for viral resistance

YMDD mutants were determined by PCR followed by restriction fragment length polymorphism after the method of Chayama *et al.*[4] HBV mutants resistant to entecavir were examined at the baseline and sequentially while patients received entecavir. HBV-DNA was extracted from the serum and amplified by PCR, and nucleotides corresponding to amino acids 1–344 of the reverse transcriptase were sequenced directly by the dideoxy-chain method of Sanger *et al.*[17]

Treatment with entecavir

The 19 patients were randomized to receive two different regimens of entecavir in a double-blind study. Thus, 0.5 and 1.0 mg entecavir was given daily to 10 and nine patients, respectively, for the first 52 weeks. Thereafter, patients in both groups received 1.0 mg/day entecavir, and the treatment was continued for an additional 68–92 weeks (120–144 weeks in total).

Response to entecavir

Biochemical response was defined by the normalization of serum alanine aminotransferase (ALT; < 50 IU/L in our laboratory), virological response by the disappearance of HBV-DNA from serum detectable by Amplicor (sensitivity, < 2.6 LGE/mL), and histological response by a decrease in histology activity index (HAI) score of 2 points or more. Necroinflammatory activity and fibrosis were evaluated by the METAVIR score as well.

Statistical analysis

Frequencies were compared between groups by the Mann–Whitney *U*-test and Fisher's exact test, and medians by the Wilcoxon signed rank test. Normalization in ALT levels and loss of HBV-DNA from the serum, as well as the development of entecavir-resistant HBV mutants, were compared by the method of Kaplan–Meier, and differences were evaluated by the log–rank test with use of the production limit method. $P < 0.05$ was considered significant. Analysis of data was performed with SPSS software (SPSS, Chicago, IL, USA).

Results

Comparison of baseline characteristics between patients given 0.5 and 1.0 mg entecavir daily for 52 weeks and then 1.0 mg for an additional 68–92 weeks

Table 1 compares demographic, biochemical, hematological and virological characteristics between 10 and nine patients with chronic hepatitis B who were randomized to receive 0.5 and 1.0 mg entecavir, respectively, daily for the initial 52 weeks. Thereafter, they all received 1.0 mg entecavir daily for an additional 68–92 weeks (120–144 weeks in total). There were no differences in age, sex, pretreatment ALT levels, platelet counts, frequency of HBeAg, distribution of HBV genotypes, HBV-DNA levels and types of YMDD mutants between the two groups of patients.

Normalization of ALT and loss of HBV-DNA from the serum in patients who received long-term entecavir treatment

Figure 1 a depicts ALT levels in 10 and nine patients who received 0.5 and 1.0 mg entecavir daily, respectively, during the initial 52 weeks; thereafter, they all received 1.0 mg entecavir daily for an additional 68–92 weeks (120–144 weeks in total). In both groups, ALT levels increased slightly during 2–4 weeks after the start of entecavir therapy, and then decreased sharply. ALT levels were lowered within the upper limit of normal (≤ 50 IU/L) 12 and 8 weeks after the start of 0.5 and 1.0 mg entecavir daily, respectively. After then, ALT levels decreased and stayed within the

Table 1 Patients with breakthrough hepatitis induced by lamivudine-resistant hepatitis B virus (HBV) mutants who were treated with two doses of entecavir during the initial 52 weeks

		Initial daily dose of entecavir	
	Total ($n = 19$)	0.5 mg ($n = 10$)	1.0 mg ($n = 9$)
Duration of entecavir (weeks)	120–144	120–144	124–140
Age (years)	38 (29–65)	37 (29–65)	39 (30–49)
Men	17 (89%)	9 (90%)	8 (89%)
ALT (IU/L)	119 (46–1708)	111 (46–1708)	275 (49–442)
Platelets ($\times 10^3/mm^3$)	190 (93–265)	180 (93–235)	190 (108–265)
HBeAg	14 (74%)	7 (70%)	7 (78%)
Genotypes (A : B : C)	1:0:18	1:0:9	0:0:9
HBV-DNA (LGE/mL)	7.2 (5.2–8.6)	7.2 (5.2–8.6)	6.6 (5.7–8.2)
YMDD mutants (I : V : I/V)	11:3:5	6:2:2	5:1:3

Median values are shown with the range in parentheses, and the ratio of HBV genotypes, as well as YIDD, YVDD and both YMDD mutants, is indicated. ALT, alanine aminotransferase; HBeAg, hepatitis e antigen; LGE, log geometric equivalents.

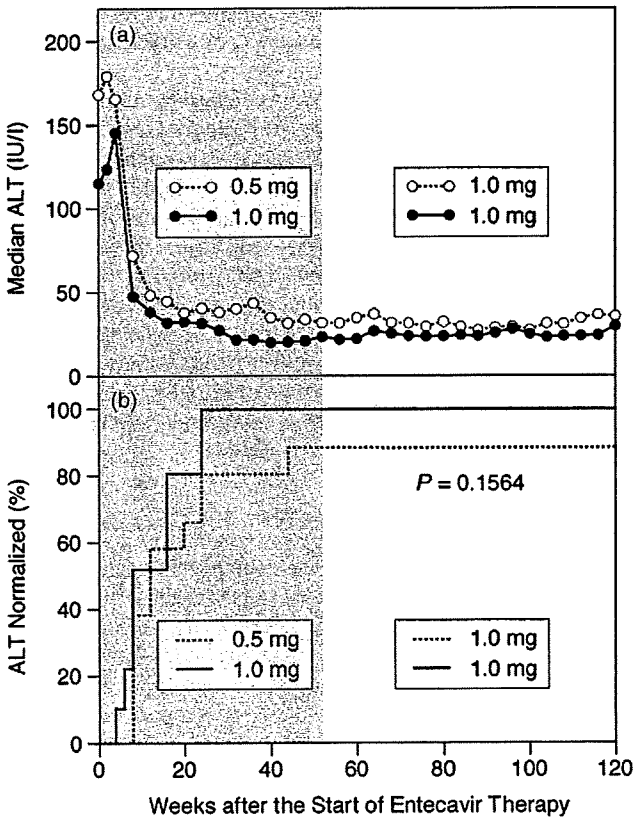

Figure 1 Alanine aminotransferase (ALT) levels in the 19 patients with breakthrough hepatitis induced by lamivudine-resistant hepatitis B virus mutants who received entecavir for 120 weeks. Of them, 10 patients received 0.5 mg and the remaining nine 1.0 mg entecavir daily during the initial 52 weeks (shaded), and thereafter both groups received 1.0 mg entecavir daily. The mean ALT levels (a) and the normalization of serum ALT (≤ 50 IU/L) (b) are illustrated.

normal limit among patients in both groups. Collectively in the 19 patients, the ALT level was normalized in 47% at week 12 and in 83% at week 24. Figure 1(b) compares the normalization of ALT levels between patients who received 0.5 and 1.0 mg entecavir daily during the initial 52 weeks. There were no statistical differences in the normalization of ALT levels between patients given 0.5 and 1.0 mg entecavir. Of the 14 patients positive for HBeAg at the start of entecavir, two (14%) lost HBeAg and seroconverted to anti-HBe, while HBsAg was not cleared from the serum in any of the 19 patients.

The loss of HBV-DNA from serum was compared between patients given 0.5 and 1.0 mg entecavir daily during the initial 52 weeks. A sharp decrease in HBV-DNA by more than 2 logs was achieved at 4 weeks in patients given the initial 0.5 mg entecavir daily, and at 8 weeks in those receiving the initial 1.0 mg entecavir daily. Twenty-four weeks after the start, HBV-DNA levels stabilized and stayed approximately 1 log lower in the patients with the initial 0.5 than 1.0 mg entecavir daily. The loss of HBV-DNA detectable by the quantitative method varied in patients with two different initial entecavir doses. At 24 weeks after the start of entecavir therapy, HBV-DNA became undetectable in 20% and 11%, respectively, of the patients with the initial 0.5 and 1.0 mg entecavir daily; the loss increased to 50% and 33% at 120 weeks, respectively. However, there were no significant differences in the loss of HBV-DNA between the patients receiving 0.5 and 1.0 mg entecavir daily during the initial 25 weeks.

Improvement of liver histology in the patients who were switched to entecavir after the development of breakthrough hepatitis during long-term lamivudine treatment

Of the 19 patients switched to receive entecavir, 12 (63%) underwent serial liver biopsies at the baseline and annually for 3 years while they were treated with entecavir. METAVIR scores for fibrosis stages at the start of entecavir were: F1 in six (50%) patients; F2 in three (25%); and F3 in three (25%). Activity grades were: A1 in six (50%) patients and A2 in six (50%). After they had received entecavir for 1 year, the fibrosis stage improved in two (17%), was

Table 2 Improvement in histology activity scores after entecavir during 3 years in the 12 patients who had developed breakthrough hepatitis induced by lamivudine-resistant HBV mutants

Features		Before	After	Decrement	Differences (*P*-value)
Periportal and/or bridging necrosis	Median (range)	1 (0–3)	0 (0–1)	1 (0–3)	0.003
	Mean ± SD	1.2 ± 0.9	0.1 ± 0.3	1.1 ± 0.8	
Lobular degeneration and focal necrosis	Median (range)	2 (0–3)	1 (0–1)	1 (0–2)	0.014
	Mean ± SD	2.0 ± 1.0	0.9 ± 0.3	1.0 ± 1.0	
Portal inflammation	Median (range)	1 (0–3)	1 (0–1)	1 (0–2)	0.015
	Mean ± SD	1.8 ± 1.0	0.8 ± 0.4	0.9 ± 0.9	
Fibrosis	Median (range)	2 (1–3)	1 (1–3)	0 (0–2)	0.059
	Mean ± SD	2.0 ± 1.0	1.4 ± 0.8	0.5 ± 1.1	
Total HAI score	Median (range)	6 (3–12)	3 (2–5)	3 (1–8)	0.002
	Mean ± SD	7.0 ± 2.7	3.2 ± 0.9	3.5 ± 2.4	

HAI, histology activity index; SD, standard deviation.

unchanged in nine (75%), and worsened in the remaining one (8%). The activity grade improved in nine (75%) patients and was unchanged in the remaining three (25%); it did not worsen in any patient.

One of the 12 patients could not receive liver biopsy 3 years after the start of therapy, because entecavir-resistant HBV mutants developed. Table 2 summarizes changes in HAI scores in the 11 patients who had received long-term entecavir treatment. After 3 years on entecavir therapy, improvement in HAI scores by 2 points or greater was achieved in nine (82%) of the 11 patients. Significant improvement was gained in the total HAI score, as well as scores for periportal and/or bridging necrosis, lobular degeneration/focal necrosis, and portal inflammation. Fibrosis score did not improve significantly ($P = 0.059$); it increased in two patients.

Clinical and virological courses of the representative patient are illustrated in Figure 2 and histological findings in yearly biopsies in Figure 3. The patient developed resistance to lamivudine and was switched to IFN. Hepatitis was exacerbated in him, however, and he was started on lamivudine again. IFN was given intermittently to him when ALT levels were elevated. Because he did not respond to IFN, entecavir was given to him. At that time, he had a HBV-DNA level of more than 7.6 LGE/mL and an HAI score of 8 in the liver biopsy. Soon after entecavir was started, HBV-DNA levels decreased sharply along with the normalization of ALT levels. He seroconverted from HBeAg to anti-HBe 1 year after the start of entecavir treatment. Histological improvement, increasing in parallel with the duration of entecavir treatment, was demonstrated by yearly liver biopsies in comparison with the baseline findings (Fig. 3). Necroinflammatory signs decreased remarkably along with narrowed portal areas, although the stage of fibrosis did not improve appreciably.

HBV mutants resistant to entecavir

Figure 4 illustrates the development of entecavir-resistant HBV mutants that increased in parallel with the duration of treatment. Entecavir-resistant HBV mutants developed in three of the 10 (30%) patients by 18, 84 and 120 weeks; and two of the nine (22%) patients by 132 and 148 weeks, respectively, who received 0.5 and 1.0 mg entecavir daily during the first year; thereafter, they all were given 1.0 mg entecavir daily for the next 68–92 weeks. During the initial 130 weeks (~2.5 years), therefore, entecavir-resistant HBV mutants developed in three of the 10 (30%) patients with the initial entecavir dose of 0.5 mg daily, in remarkable contrast to no emergence of such mutants in any of the nine patients that received 1.0 mg daily.

Alanine aminotransferase levels were elevated in only two of the five (40%) patients infected with entecavir-resistant HBV mutants, however. These two patients were switched to receive adefovir in combination with lamivudine, and breakthrough hepatitis resolved in them both. All the five patients who developed entecavir-resistant HBV mutants had been infected with lamivudine-resistant YMDD mutants with M204V in the presence or absence of M204I. In outstanding contrast, entecavir-resistant HBV mutants did not develop in any of the 11 patients who had been infected with YMDD mutants with M204I alone.

No adverse effects developed in any of the 19 patients. Breakthrough hepatitis occurred in only one of the five (20%) patients in whom entecavir-resistant mutants emerged.

Discussion

We have previously reported in the Journal that entecavir suppresses serum HBV-DNA to undetectable levels and normalizes ALT levels in more than 30% , respectively, in lamivudine-resistance patients with chronic hepatitis B at 52 weeks.[12] In the present report, we have followed 19 patients from one of the participating centers for 3 years so as to establish longer-term histological efficacy and rates of viral resistance with entecavir treatment of lamivudine-resistant chronic hepatitis B.

As in the earlier report,[12] among the 19 patients described here, ALT levels were normalized in more than 90% of them 8–12 weeks after the start of entecavir until the end of treatment. Although the median HBV-DNA level dropped by 3 logs and remained low during the entecavir therapy, they became undetectable in only 20–40% of the 19 patients. In a previous report, also, the loss of detectable HBV-DNA from the serum was achieved in only 27 of the 141 (19%) patients with lamivudine-resistant HBV mutants after they had received 1.0 mg entecavir daily for 52 weeks.[14] In a remarkable contrast, entecavir is much more efficient in treatment-naive patients who had received it for 1–2 years; HBV-DNA disappeared from the serum in 67–90% of them.[10,11,18] These differences could be attributed to some lamivudine-resistant

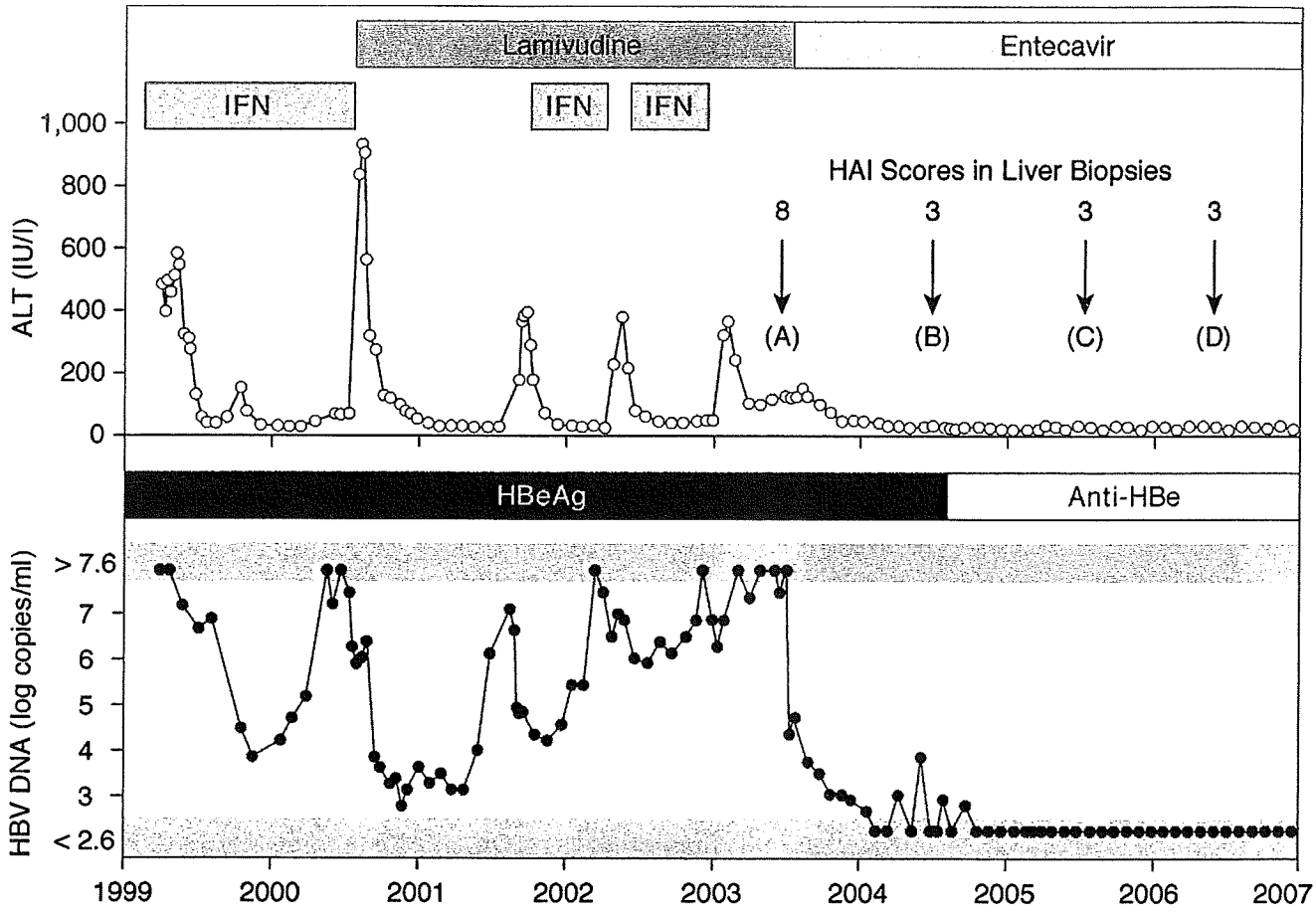

Figure 2 Clinical course of the representative patient. Fluctuating levels of alanine aminotransferase (ALT) and hepatitis B virus (HBV)-DNA are illustrated. Antiviral treatments as well as duration of hepatitis B e antigen (HBeAg) and anti-HBe are indicated by horizontal bars. Also given are time points when four liver biopsies were undertaken, along with histological activity index scores on the top. IFN, interferon.

HBV mutants contributing to the development of entecavir-resistance.[13,18]

Entecavir is a cyclopentyl guanosine analog and can inhibit the polymerase of hepadnaviridae selectively by interfering with priming and reverse transcription, as well as synthesis of minus- and plus-stranded HBV-DNA species.[19] In an *in vitro* expression system with HepG2 cells, entecavir exhibited an antiviral activity with EC_{50} of 0.00375 μM, which is 1500-fold higher than 10 μM of lamivudine.[20] Dose-dependent pharmacological activity of entecavir was evident in a randomized double-blind trial.[21] Although 0.01 mg entecavir daily decreased HBV-DNA by 2.41 logs at 22 weeks, the antiviral activity was significantly lower than 4.31 and 4.72 logs, respectively, of 0.1 and 0.5 mg daily; they were both higher than 3.36 logs by 100 mg lamivudine daily, however. Accordingly, normalization of ALT was more frequent by treatments with 0.1 and 0.5 mg entecavir daily (69% and 83%, respectively) than with 100 mg lamivudine daily (59%).

Significant decrease in HAI scores has been reported in patients with chronic hepatitis B who had received lamivudine for 1–3 years.[22,23] Furthermore, decreases in hepatic inflammation may improve the fibrosis stage. Entecavir therapy for 52 weeks has achieved histological improvement in 55–72% of patients in phase III clinical trials.[10,11,14] In corroboration of these results, fibrosis stage and inflammation grade improved in the present series of patients who had received entecavir for 3 years, with a significant decrease in the HAI score (Table 2). Histological improvement would have been gained by long-term entecavir therapy, and it may further increase, should entecavir be continued further.

Long-term entecavir treatment, however, may be hampered by the development of drug-resistant mutants. Although entecavir-resistant HBV mutants rarely occur in treatment-naive patients,[18] they can emerge rather frequently in the patients infected with lamivudine-resistant HBV mutants.[14,24] In the present study, entecavir-resistant HBV mutants developed in five of the 19 (26%) lamivudine-resistant patients during 144 weeks of treatment. The incidence was comparable with 32% in the lamivudine-resistant patients who had received entecavir for 3 years.[24] Only two (40%) of them developed hepatitis flares and they were switched to receive adefovir in combination with lamivudine. Entecavir-resistant HBV mutants emerging in patients with lamivudine-resistant mutants are reported to be replication-impaired and rarely induce breakthrough hepatitis.[25] It should be found out how entecavir-resistant HBV mutants can be managed with antiviral nucleos(t)ide analogs other than lamivudine and entecavir, or

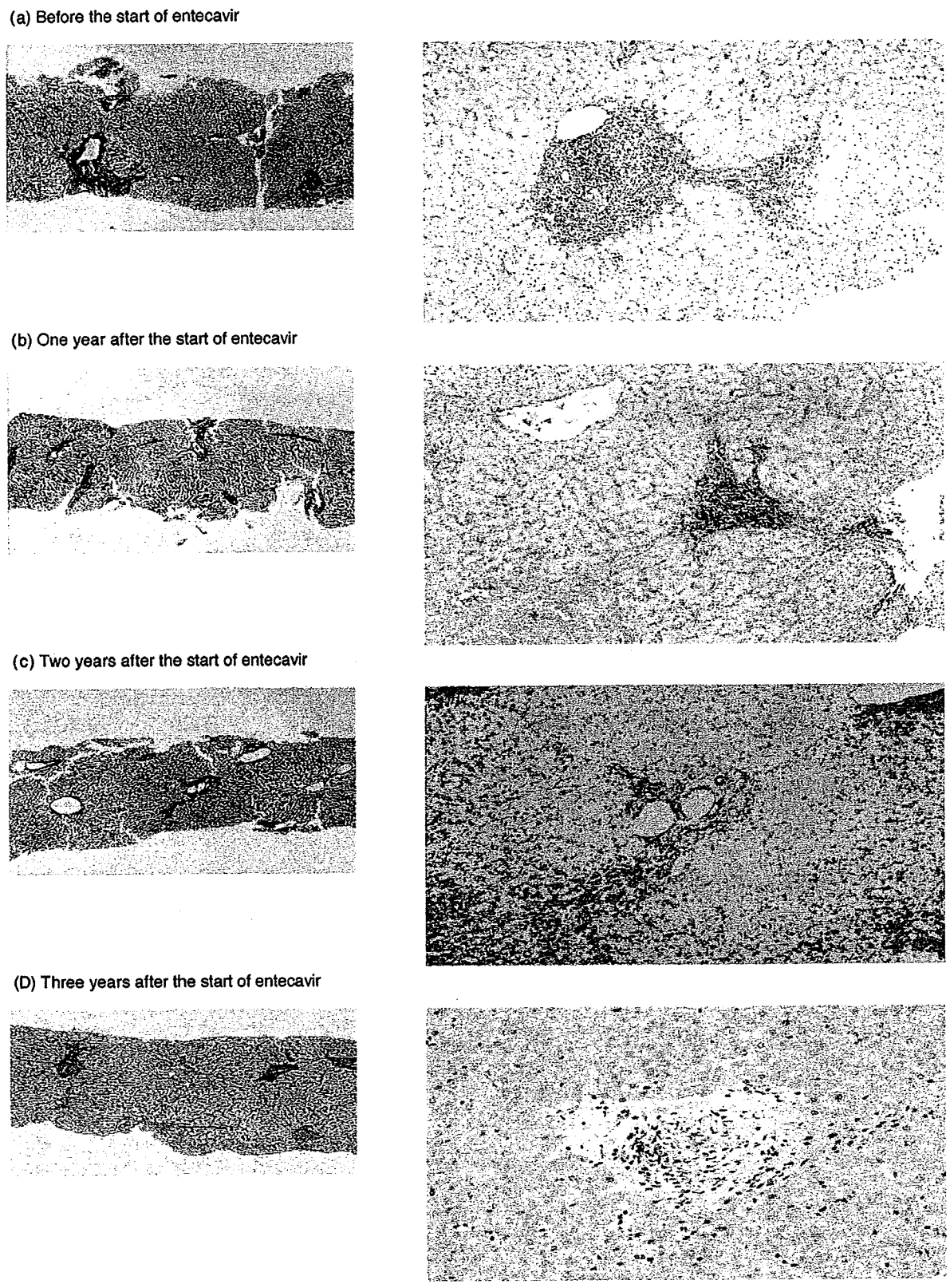

Figure 3 Histological changes in the representative patient during 3-year entecavir treatment (Fig. 2). With hematoxylin–eosin stain on the left, marked enlargement of portal areas is evident along with infiltration of mononuclear cells before the switch from lamivudine to entecavir (a). They decreased increasingly during the 3-year treatment with entecavir (b–d). Stage of fibrosis did not change appreciably by the staining for silver on the right.

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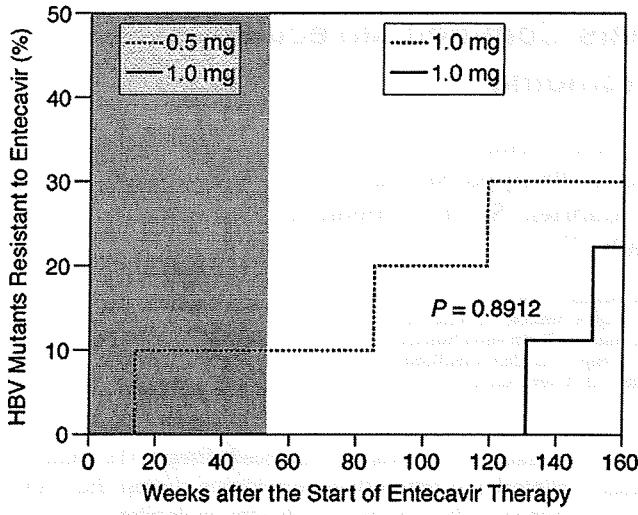

Figure 4 Development of entecavir-resistant hepatitis B virus (HBV) mutants during the 3-year treatment. The 10 patients with the initial entecavir dose of 0.5 mg daily and the nine with that of 1.0 mg daily are compared.

combination thereof. It has been proposed that adefovir add-on lamivudine is efficacious with negligible drug resistance over 3 years.[26,27]

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