**Fig. 5** Images in a female patient who had hepatocellular carcinoma (HCC) in segment VI. Before treatment, scans obtained on computed tomography during hepatic arteriography (CTHA) (**a**) and computed tomography during arterial portography (CTAP) (**b**) showed a nodular HCC (*arrow*) measuring 2.5 cm. Three applicators were placed in parallel in the HCC in liver segment VI, and then the tumor was ablated in one procedure (total ablation time 13 min 42 s, total applied energy 35.3 kJ). After the procedure, computed tomography (CT) images showed a necrotic area of 46 mm in diameter including the nodular HCC (**c** [*arrows* show applicator for insertion paths], **d** [*arrow* shows applicator for insertion paths])
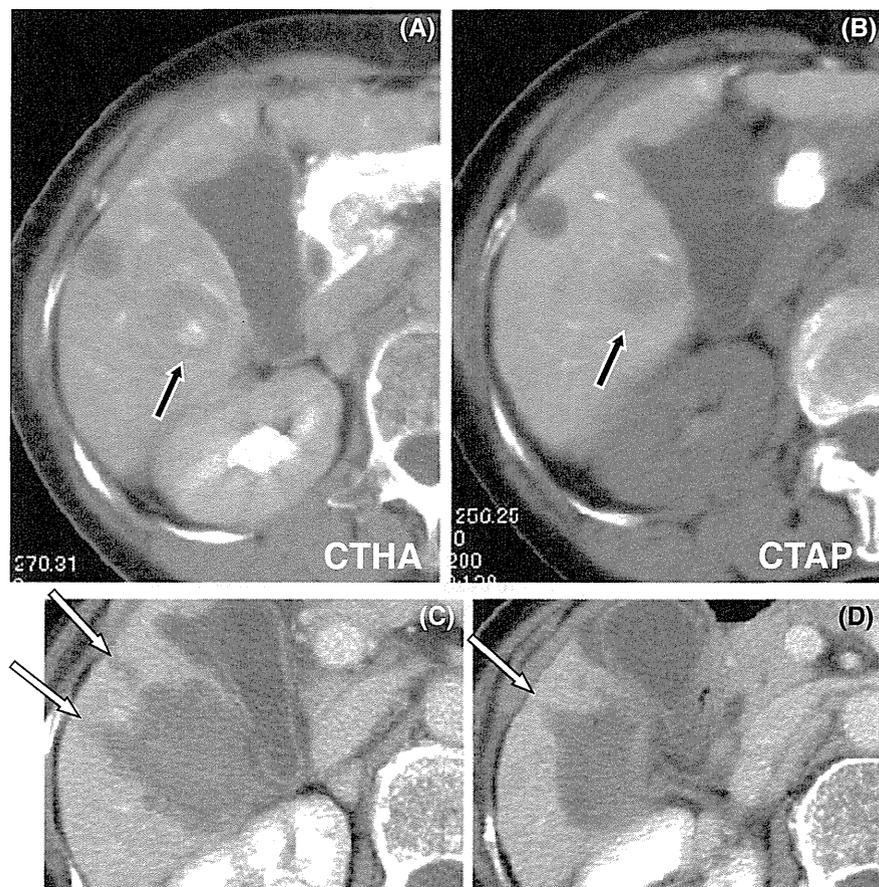
Table 2 Maintenance of the therapeutic effect (TE) (overall assessment of the TE; intention-to-treat (ITT) analysis)

	This clinical study		Patients who underwent local therapy [24]	
	10 weeks	24 weeks[a]	3 months	6 months
Complete response (CR) (no. of patients)	85/88 (96.6 %)	82/87 (94.3 %)	4468/5394 (82.8 %)	4318/5378 (80.3 %)
Other (no. of patients)	3/88 (3.4 %)[b]	5/87 (5.7 %)[c]	926/5394 (17.2 %)	1060/5378 (19.7 %)

[a] One patient who died was omitted from the 24-week assessment

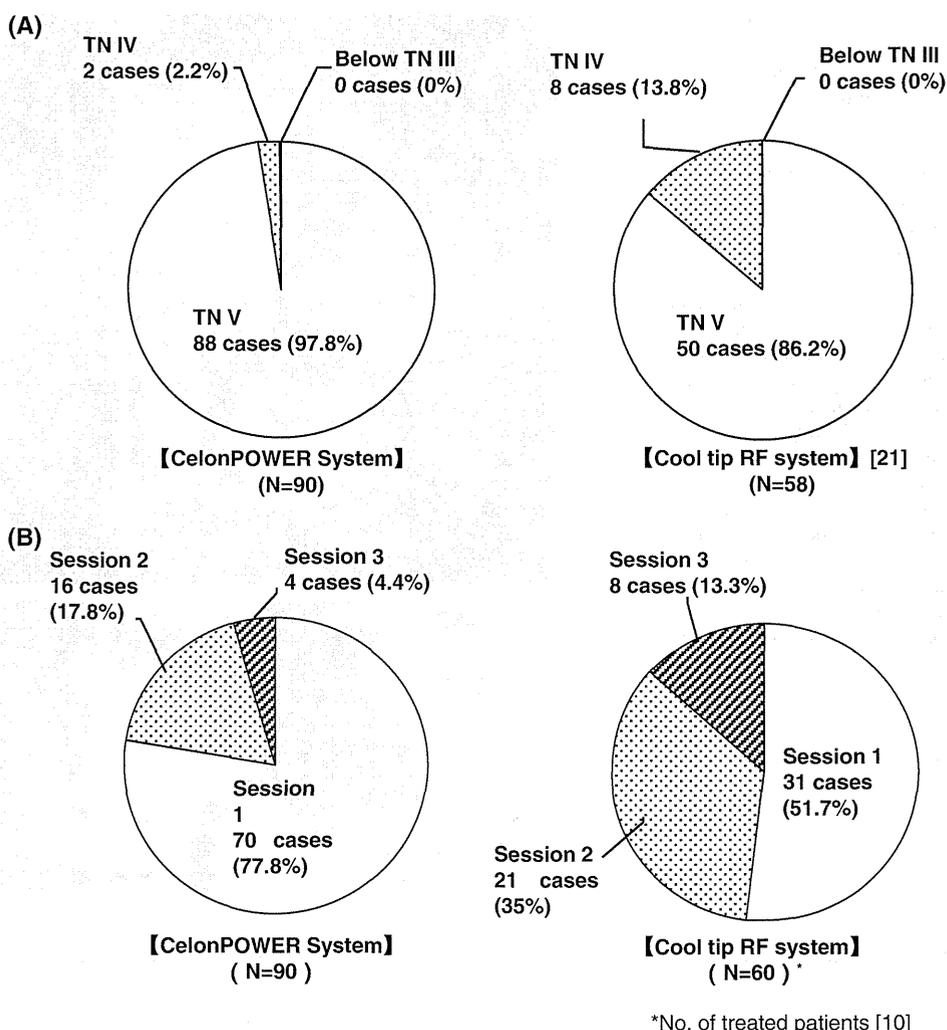[b] Includes 3 patients who developed local recurrence within 10 weeks

[c] Includes 5 patients who developed local recurrence within 24 weeks

of each RFA session in the present clinical study. As shown in Fig. 6a, the complete necrosis (Class V TN) rate with the CelonPOWER System was 97.8 % (88/90 patients), which was higher than the rate of 86.2 % (50/58 patients) with the Cool-tip RF System. These results thus confirm the non-inferiority of the CelonPOWER System ($p < 0.001$; Fisher's exact test based on binomial distribution). As shown in Fig. 6b, the percentage of patients in whom treatment was completed in a single session was 77.8 % (70/90 patients) in the present study with the CelonPOWER System, compared with 51.7 % (31/60 patients) in the Cool-tip RF System study [10].

Safety

The overall safety assessment was performed for the entire clinical study period, i.e., inclusive of the treatment period and the follow-up period. Of the 91 patients included in the safety analysis, no procedure was rated as unsafe, although 2 procedures (2.2 %) were rated as somewhat unsafe, one with an abdominal wall burn and one with biliary peritonitis owing to bile leakage; 78 procedures (85.7 %) were rated as safe overall and 11 procedures (12.1 %) were rated as safe. There was no device failure. In the patient with biliary peritonitis, three 30-mm electrodes had been

Fig. 6 Comparison of the present results obtained with the CelonPOWER System and the clinical study results reported for the Cool-tip RF System. The percentage of Class V tumor necrosis (*TN*) (TN 100 %) cases (a) and the number of patients in whom each RFA session was completed (b)

(A)

TN IV 2 cases (2.2%)
Below TN III 0 cases (0%)
TN V 88 cases (97.8%)

【CelonPOWER System】
(N=90)

TN IV 8 cases (13.8%)
Below TN III 0 cases (0%)
TN V 50 cases (86.2%)

【Cool tip RF system】 [21]
(N=58)

(B)

Session 2 16 cases (17.8%)
Session 3 4 cases (4.4%)
Session 1 70 cases (77.8%)

【CelonPOWER System】
(N=90)

Session 3 8 cases (13.3%)
Session 1 31 cases (51.7%)
Session 2 21 cases (35%)

【Cool tip RF system】
(N=60) *

*No. of treated patients [10]

simultaneously inserted into an S8 tumor, and treatment was finished in a single ablation.

During the course of the entire clinical study period, serious adverse events (i.e., events for which a causal relationship with the CelonPOWER System could not be ruled out) were seen in 3 patients, consisting of abdominal wall burn, pleural effusion, and biliary peritonitis. Each of those events was judged to be serious because they required prolongation of hospitalization, and each required treatment. In addition, it was judged that each of these serious adverse events was a known adverse event that had been observed with similar, already-approved RFA devices [21–23]. Also, the single fatality, which occurred at home, had occurred in a patient who had been hospitalized for treatment on the suspicion of peritonitis based on the examinations performed after 10 weeks in the follow-up period. The patient's condition had improved and the patient had been discharged, and it was later confirmed that death had occurred at home. Autopsy revealed the cause of death to have been due to the progression of cirrhosis, and

it was thus thought that the death was not related to the treatment with the CelonPOWER System. Table 3 shows the most common adverse effects (those observed in 5 % of patients or more) and all of these (pleural effusion, nausea, vomiting, postprocedural pain, and fever) have been known to occur with previously approved local therapeutic devices. Moreover, all the adverse events were easily controllable.

Discussion

We set out to prospectively determine whether a bipolar RFA device (CelonPOWER System) was safe and effective in the treatment of liver cancer and whether it could be demonstrated to be non-inferior to a monopolar RFA system currently approved and employed clinically in Japan (Cool-tip RF System).

Treatment was completed in a fewer number of sessions when using the CelonPOWER System than with the Cool-

Table 3 Frequently observed adverse effects (5 % or more) (adverse reactions at an incidence of >5 % in the overall study period)

Adverse event	No. of patients	%	No. of patients treated (%)	Treatments
Aspartate aminotransferase (AST) increase	72	79.1	0 (0)	–
Alanine aminotransferase (ALT) increase	69	75.8	0 (0)	–
Lactate dehydrogenase (LDH) increase	22	24.2	0 (0)	–
Total bilirubin increase	20	22.0	0 (0)	–
Pleural effusion	12	13.2	2 (2.20)	Human serum albumin, cefmetazole sodium, tazobactam piperacillin hydrate
Vomiting	12	13.2	7 (7.69)	Metoclopramide
Nausea	10	11.0	9 (9.89)	Metoclopramide, domperidone, diazepam
Postoperative pain	9	9.9	3 (3.30)	Pentazocine, loxoprofen sodium hydrate, acetaminophen, diclofenac sodium
White blood cell count increase	8	8.8	1 (1.10)	Sulbactam sodium–cefoperazone sodium
Platelet count decrease	6	6.6	0 (0)	–
Alkaline phosphatase (ALP) increase	5	5.5	0 (0)	–
Fever	5	5.5	5 (5.49)	Loxoprofen sodium hydrate, acetaminophen, cefmetazole sodium

tip RF System, suggesting that this new system yields efficacy that is at least equivalent to that achieved with the Cool-tip RF System, while causing less of a treatment burden on the patient.

We assessed the TE level, and its maintenance in ITT cases after 10 weeks and 24 weeks (6 months) in the follow-up period of this clinical study and found that the overall TE assessment was not inferior to that of the National Follow-up Survey Report on Primary Hepatic Carcinoma (2004–2005) [24] issued by the Liver Cancer Study Group of Japan (Table 2). Considering that the method for overall TE assessment in that report was the same as that employed in the present study, it is reasonable to conclude that the TE maintenance with the CelonPOWER System is not inferior to that of other local therapy.

Nishikawa et al. reported on local recurrence when using monopolar systems clinically. They found that, in 269 patients with solitary hypervascular HCCs who had undergone RFA, the 1- and 2-year cumulative local recurrence rates were 12.8 and 23.6 %, respectively [25]. We believe that our present results for the cumulative local recurrence rate (5.7 % for 6 months) with the Celon-POWER System are comparable to those reported results.

The introduction of a new device inevitably raises the question of its safety. In our series, there were 3 adverse events—one event of abdominal wall burn and one of pleural effusion during the treatment period, and one event of biliary peritonitis during the follow-up period. These adverse events were previously known to be possible adverse events that had been observed with the Cool-tip, RITA, and Boston monopolar RFA systems that have already been approved for clinical use in Japan [21–23]. Therefore, similar caution concerning internal adverse events is necessary when using the CelonPOWER System, although the problem of external burns does not exist with this system.

The high-incidence (≥5 %) device-related adverse event rate during the course of our clinical study was similar to the rates with the Cool-tip, RITA, and Boston monopolar RFA systems [21–23].

Therefore, these events are not unique to the Celon-POWER System, and the safety of the CelonPOWER System is not inferior to that of the existing approved RFA devices.

This study has several limitations. First of all, although it was a prospective study, it was not a randomized controlled clinical study. However, all consecutive patients who satisfied the enrollment criteria were offered the opportunity to participate and the study was performed in all those who provided informed consent and decided to receive the treatment. After providing informed consent, 5 patients decided not to participate and 1 ceased treatment after 1 session, due to the difficulty posed by the proximity of the lesion to the heart and lungs.

Although we were able to compare our own results immediately after treatment with those of the Cool-tip RF

System and other systems, we were not able to compare the results 6 months after treatment because of the lack of such data for the Cool-tip RF System, because of the different GCP guidelines in force at the time of the Cool-tip RF System study. However, the 6-month follow-up data of our study were very satisfactory. Furthermore, because there were no such data available in the reports on the Cool-tip RF System, we could not compare the levels of experience of the operators in the two studies.

In conclusion, the present clinical study confirmed that the CelonPOWER System is a very safe and highly effective RFA system for liver cancer in Japanese patients. In addition, because this system is a bipolar device, it operates with high energy efficiency, and because multiple multipolar applicators can be employed simultaneously, coagulation necrosis of an extensive tumor tissue volume can be achieved in a short treatment time. Moreover, throughout the course of this clinical study, most of the patients did not experience hot flushes or perspiration. It is therefore anticipated that the CelonPOWER System will become used as a next-generation RFA system that is not only safer than existing systems, but is highly effective and places less physical burden on the patient.

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Long-Term Entecavir Treatment Reduces Hepatocellular Carcinoma Incidence in Patients With Hepatitis B Virus Infection

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Chronic hepatitis B virus (HBV) infection leads to cirrhosis and hepatocellular carcinoma (HCC). Antiviral agents are thought to reduce HCC development, but agents such as lamivudine (LAM) have a high rate of drug resistance. We compared the incidence of HCC in 472 entecavir (ETV)-treated patients and 1,143 nontreated HBV patients (control group). Propensity score matching eliminated the baseline differences, resulting in a sample size of 316 patients per cohort. The drug mutation resistance was 0.8% (4/472) in the ETV group. The cumulative HCC incidence rates at 5 years were 3.7% and 13.7% for the ETV and control groups, respectively ($P < 0.001$). Cox proportional hazard regression analysis, adjusted for a number of known HCC risk factors, showed that patients in the ETV group were less likely to develop HCC than those in the control group (hazard ratio: 0.37; 95% confidence interval: 0.15-0.91; $P = 0.030$). Both cohorts were applied in three previously reported risk scales and risk scores were generated based on age, gender, cirrhosis status, levels of alanine aminotransferase, hepatitis B e antigen, baseline HBV DNA, albumin, and bilirubin. The greatest HCC risk reduction occurred in high-risk patients who scored higher on respective risk scales. In sub analyses, we compared treatment effect between nucleos(t)ide analogs, which included matched LAM-treated patients without rescue therapy (n = 182). We found HCC suppression effect greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) cirrhosis patients when they were compared with the control group. *Conclusion:* Long-term ETV treatment may reduce the incidence of HCC in HBV-infected patients. The treatment effect was greater in patients at higher risk of HCC. (HEPATOLOGY 2013;58:98-107)

More than 2 billion people worldwide have been exposed to hepatitis B virus (HBV) and about 350 million people are chronically infected, the majority of whom are in Asia (75%). The prevalence of HBV in Japan is 0.8%, which is lower than other Asian countries such as Taiwan (>10%) and China.[1-3] As chronic HBV infection leads to cirrhosis and hepatocellular carcinoma (HCC), published studies have shown that up to 25% of chronically infected patients eventually die of liver cirrhosis or HCC.[4]

A large-scale longitudinal epidemiologic study has shown that a patient's baseline HBV DNA level is an independent predictor for the development of HCC.[5] Studies have begun to show that treatment to decrease HBV DNA reduces the risk of HCC development in HBV patients with cirrhosis or advanced fibrosis or in chronic HBV patients.[6,7]

Within the past 10 years, new antiviral therapies, including nucleos(t)ide analogs (NAs), have been approved and were successful in suppressing circulating serum viral loads. Studies that have examined the relationship between NA therapy and HCC almost exclusively used older drugs such as lamivudine and/or adefovir. Although results of long-term studies showed the importance of antiviral suppression, HCC risk among patients treated by newer NAs remains inconclusive. Entecavir (ETV) is a relatively new antiviral NA that has proved effective in suppressing HBV

Entecavir group

Consecutive 510 patients began to receive ETV from 2004 to 2010

exclude

→ Follow-up duration < 1yr, 28pts

482 patients received ETV for more than 1 year

→ Adequate clinical data and frozen serum samples missing, 10pts

472 pts mono-infected with HBV enrolled as ETV treatment cohort

Control group

Consecutive 2332 patients with chronically infected HBV began to be followed up before 1999

exclude

→ Follow-up duration < 1yr, 262 pts

2070 patients followed up for more than 1 year

→ Administrated steroid after follow-up, 120 pts
Administrated IFN after follow-up, 305 pts
Administrated NA after follow-up, 273 pts

1372 patients did not receive antiviral therapy

→ Adequate clinical data and frozen serum samples missing, 153 pts

1219 patients with adequate clinical data and frozen serum samples at enrollment

→ HCV-Ab positive, 76 pts

1143 pts mono-infected with HBV enrolled as the historical control cohort without antiviral therapy

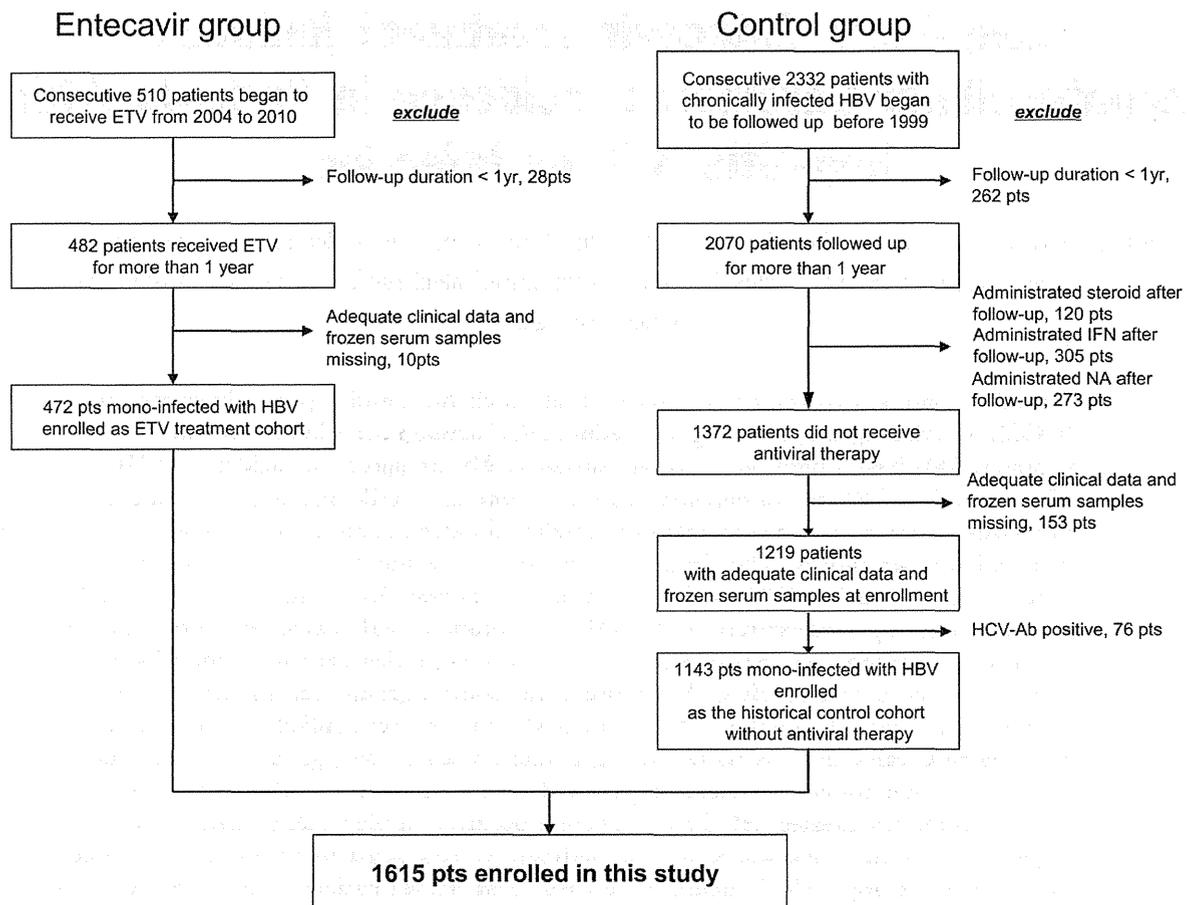**1615 pts enrolled in this study**

Fig. 1. Entecavir-treated and nontreated cohorts. ETV, entecavir; HBV, hepatitis B virus; IFN, interferon; NA, nucleos(t)ide; HCV-Ab, anti-hepatitis C virus antibody.

DNA replications with minimal drug resistance.[8,9] In this study we examined whether long-term ETV treatment would reduce HCC risk in HBV-infected patients when compared with NA-naïve patients.

Patients and Methods

Patients and Design. From 2004 to 2010, we consecutively recruited 510 patients treated with 0.5 mg ETV (ETV group); the ETV group was compared with a retrospective cohort of 2,332 NA-naïve, HBV-infected patients (control group).

These patients were chronically monoinfected with HBV and were confirmed as hepatitis B s antigen (HBsAg)-positive for at least 6 months. As a general rule,

ETV was initiated in a patient who had both abnormal alanine aminotransferase (ALT) levels (defined as ALT ≥45) and elevated HBV DNA levels of ≥4 log copies/mL. A patient with advanced fibrosis would be treated with ETV if the ALT level was normal; however, a patient without fibrosis or with a normal HBV DNA/ALT level would not be treated with ETV. Among the treated patients, 38 were excluded from the ETV group either because their follow-up period was less than 1 year (n = 28) or because the clinical data or serum samples were incomplete (n = 10). The remaining 472 ETV-treated patients were included in the analysis (Fig. 1). No patient in the ETV group received other NAs before ETV treatment.

The control group patients were recruited from 1973 to 1999. These patients were NA-naïve at baseline, as no NA therapy had yet been approved. Patients were excluded from the control group if (1) their follow-up duration was less than 1 year (n = 262); (2) corticosteroid withdrawal therapy (n = 120), IFN treatment (n = 305) or NA treatment (n = 273) was initiated during follow-up; (3) clinical data or serum samples were incomplete (n = 153); or (4) patients were found to be positive for anti-hepatitis C virus antibodies (HCV-Ab) (n = 76). The remaining 1,143 patients served as the control population (Fig. 1).

We also made subanalyses to examine the difference of HCC suppression effect between NAs. To make this comparison, we recruited a cohort of 949 consecutive patients from our hospital who were treated with lamivudine (LAM) (September 1995 to September 2007). LAM-treated patients who met the same inclusion criteria as the ETV group, who had no rescue therapy (LAM group, n = 492), were used in the comparison.

We received informed consent from each patient at their entry into the study. Informed consent for the clinical data collection and storage of serum samples were obtained from each patient in the historical control group. The study protocol was in accordance with the ethical guidelines of the Declaration of Helsinki and approved by the Toranomon Hospital Ethics Committee.

Clinical Data Collection and Follow-up. All ETV-treated and untreated patients were followed at 1- to 3-month intervals, during which biochemical and HBV virological markers, blood counts, tumor markers (e.g., alpha-fetoprotein and des-γ-carboxylprothrombin), and cirrhosis and HCC status were monitored. Viral response in the ETV group was defined as a reduction in HBV DNA levels to below 400 copies/mL. Cirrhosis was determined by laparoscopy, liver biopsy, imaging modalities, or portal hypertension. HCC was diagnosed predominantly via imaging, including dynamic computed tomography, magnetic resonance imaging, and/or digital subtraction angiography. When the hepatic nodule did not show typical imaging features, diagnosis was confirmed by fine-needle aspiration biopsy followed by histological examination. Patients were followed until any confirmed HCC diagnosis 1 year after the start of observation (primary outcome) or until the last visit before December 2011. All patients also underwent ultrasonography or helical dynamic computed tomography every 3 to 6 months (cirrhosis patients) or every 6 to 12 months (noncirrhosis patients).

HBV Infection Markers. HBV DNA levels were quantified using the COBAS Amplicor HBV Monitor Test (Roche Diagnostics, Tokyo, Japan), which has a dynamic range of 2.6 to 7.6 log copies/mL, or COBAS TaqMan HBV Test v2.0 (Roche Diagnostics) which has a dynamic range of over 2.1 to 9.0 log copies/mL. HBV DNA of the control group was measured from their stored frozen serum (−80°C) using COBAS TaqMan HBV v.2.0 once at the start of observation. Previous measurements were taken using the old DNA polymerase assay in the control group and thus were not used for comparisons. For the ETV group, drug-resistant mutations were determined from a nested polymerase chain reaction, using a primer specific at the polymerase region in patients who had an HBV DNA relapse of ≥ 1 log copies from nadir. Hepatitis B e antigen; (HBeAg) was determined by enzyme-linked immunosorbent assay with a commercial kit (HBeAg EIA; Institute of Immunology, Tokyo, Japan). A commercial kit (HBV Genotype EIA; Institute of Immunology) was used to serologically determine HBV genotypes using the combination of epitopes expressed on the pre-S2 region product, which is specific for each of the eight major genotypes (A to H).

HCC Incidence by Risk Scores. To examine HCC incidence by risk scores, we applied published HCC risk scales, which are based on the natural course of HCC among HBV-positive patients, to our cohorts. We first searched Medline/PubMed using "hepatitis B," "cancer," and "risk score" as keywords and found four publications in English that used risk-score estimations.[10-13] One article was rejected because we were unable to compute the risk scores with our variables, and therefore we used only the scales indicated by the remaining three publications to generate the risk scores.[13] The risk scales were based on parameters such as age, gender, cirrhosis, levels of ALT, HBeAg, baseline HBV DNA, albumin, and bilirubin. The original risk score formula and the risk score distributions for our two cohorts derived from these formulas are shown in Supporting Table 1. The risk score cutoff points were determined from the following original articles. In Yang et al.'s article,[10] the risk score was derived from 17-point categories. When we applied the scores to our control group, we found that the 12-point scale was at best in detecting a difference in HCC incidence. With that, we examined the HCC suppression treatment effect by dividing the patients into equal halves with 12 points as the cutoff. Yuen et al.[11] divided their cohort in half and found risk scores of 82 as the optimal cutoff point. We also applied the same cutoff point to our cohorts. Wong et al.[12] used their risk scores to categorize their cohort into low-risk, medium-risk, and high-risk groups with respective cutoff points at <4, 4-19, ≥ 20. We also applied the same cutoff points to our cohorts to examine the treatment effect. Cumulative

HCC incidence rates were compared by these risk scores between the ETV and control groups.

Statistical Analysis. Categorical data were compared using chi-square or Fisher's exact tests. Continuous variables with normal distributions were compared using Student's *t* test, and those without normal distributions were compared using the Mann-Whitney *U* test. Cumulative HCC incidence rates were analyzed using the Kaplan-Meier method; patients followed beyond 5 years were censored to better compare the two cohorts because the ETV group had a shorter follow-up period when compared with the historical control group. We compared the cumulative incidence of HCC using the log-rank test, and Cox proportional hazard regression analysis, which was used to assess the variables that were significantly associated with the development of HCC. Deaths before HCC development were censored. Significance was defined as $P < 0.05$ for all two-tailed tests.

We used the propensity score (PS) matching method to reduce significant differences in demographics between the ETV and control groups.[14,15] Using multiple logistic regression analysis, a PS was estimated for all patients treated with ETV.[14] Variables used in the model included age, sex, presence of cirrhosis, HBeAg, HBV DNA< aspartate aminotransferase (AST), ALT, γ-glutamyl transpeptidase; (γ-GTP), bilirubin, albumin, and platelet counts. We performed caliper matching on the PS (nearest available matching). Pairs (ETV and the control group) on the PS logit were matched to within a range of 0.2 standard deviation (SD).[16,17] The PS logit distributions for each cohort showing the overlaps and SD ranges are shown in Supporting Fig. 1. The balance of covariates was measured by their standardized differences. A difference >10% of the absolute value was considered significantly imbalanced.[17] The cohorts were divided into five PS quintiles (Supporting Table 2). We also made subanalyses to examine the difference of HCC suppression effect between NAs by comparing the HCC incidence between propensity score matched ETV- and lamivudine (LAM)-treated patients without a rescue therapy. The LAM-treated patients were derived from consecutive sampling at our institution and were PS matched with ETV group according to the same method described above. Interaction of the subgroups by pre-existing cirrhosis or risk scores and ETV treatment were evaluated. $P < 0.10$ was considered statistically significant. Data analysis was performed using IBM SPSS v. 19.0 software (Armonk, NY) and R software v. 2.13 (R Foundation for Statistical Computing, Vienna, Austria; www.r-project.org).

Results

Patient Characteristics. The patient characteristics at the baseline, before PS matching are shown in Table 1. The ETV group was followed for an average of 3.2 years (1,561 person-years), whereas the control group was followed for an average of 9.5 years (12,381 person-years). Before matching, patients in the ETV group and the control group differed significantly in age, gender, genotype, baseline HBV DNA level, and other clinical data. In the ETV group, 421 patients (89%) had HBV DNA (<400 copies/mL) at year 1. Not all patients in the control group were tested for HBV DNA level during follow-up. The drug mutation resistance was 0.8% (4/472). The four patients who had drug mutation did not develop HCC. During follow-up, 12 patients (2.54%) in the ETV group and 144 patients (12.60%) in the control group developed HCC. The incidence rates of HCC for the ETV and the control groups were 76/10,000 patient-years and 116/10,000 patient-years, respectively. During this period, 21 patients in the control group developed liver cirrhosis while no patient developed liver cirrhosis in the ETV group. During the same observation period, there were four deaths in the ETV group and 10 deaths in the control group. We took competing risk into account[18,19] and compared incidence of non-HCC deaths between the cohorts and the results were not different. However, because there were only four patients in the non-HCC deaths in the ETV group (two patients in the PS matched cohort) and 10 patients in the control group (six patients in the PS matched cohort), we considered that it was not meaningful to apply competing risk analysis in our cohorts.

Factors Associated with HCC and Effect of ETV Treatment on HCC Development. To allow a common ground for comparison between the two cohorts, we used PS matching with selected key characteristics and compared the two groups within the same time period of 5 years. The PS matching process resulted in a matched sample size that consisted of 316 patients in each group (Table 1). The PS matching reduced the significant variability of the two cohorts. While five (42%) of the 12 covariates varied by >10% before matching, all covariates differed by <10% of the absolute value after matching (Supporting Fig. 2). In the PS score matched cohort, 10 out of the 231 noncirrhosis patients progressed to liver cirrhosis within the 5 years of observation. The cumulative incidence rates of HCC in the matched ETV groups were 0.7% at year 2, 1.2% at year 3, 2.5% at year 4, and 3.7% at year 5. The cumulative incidence rates of HCC in the

Table 1. Patient Characteristics and Demographics

Characteristics	Entire Cohort All Patients (n = 1,615)	Entecavir (n = 472)	Control (n = 1,143)	P	Propensity Score Matched Cohort Entecavir (n = 316)	Control (n = 316)	P
Age (y)†	42 (13.5)	47 (12.4)	39 (13.1)	<0.001	46 (12.1)	46 (13.5)	0.907
Gender (male:female)	1,035:580	315:157	720: 423	0.171	210:106	210:106	1.000
Alcohol consumption (>200kg)	355 (22)	97 (20.5)	288 (25.1)	0.013	62 (20)	105 (33)	<0.001
Cigarette smoking	443 (27)	157 (33.2)	286 (25.0)	0.005	110 (35)	110 (35)	1.000
Preexisting cirrhosis	311 (19)	116 (25)	195 (17)	0.001	79 (25)	85 (29)	0.324
HBV genotype	–	–	–	<0.001	–	–	0.843
A	53 (3.3)	12 (2.5)	41 (3.6)	–	8 (2.5)	9 (2.8)	–
B	254 (15.7)	66 (14.0)	188 (16.4)	–	49 (15.5)	50 (15.8)	–
C	1,135 (70.3)	344 (72.9)	791 (69.2)	–	225 (71.2)	226 (71.5)	–
D	1 (0.06)	0	1 (0.09)	–	0	0	–
F	1 (0.06)	0	1 (0.09)	–	0	0	–
H	2 (0.1)	2 (0.4)	0	–	0	0	–
Unclassified / missing	169 (10.4)	48 (10.2)	121 (10.5)	–	34 (10.7)	31 (9.8)	–
Baseline HBeAg positive	617 (38)	219 (46)	398 (35)	<0.001	135 (43)	133 (42)	0.936
Baseline HBV DNA (log copies/mL)	6.0 (4.3-7.7)	6.7 (5.3-8.0)	5.8 (4.0-7.5)	<0.001	6.3 (5.2-7.9)	6.6 (4.5-7.8)	0.795
Baseline AST level (IU/L)	35 (22-63)	53 (35-95)	28 (20-50)	<0.001	45 (32-70)	49 (27-98)	0.956
Baseline AST level (x ULN)	1.1 (0.7-1.9)	1.6 (1.1-2.9)	0.8 (0.6-1.5)	<0.001	1.4 (1.0-2.1)	1.5 (0.8-3.0)	0.989
Baseline ALT level (IU/L)	42 (22-88)	70 (42-163)	33 (20-68)	<0.001	61 (39-109)	60 (28-144)	0.110
Baseline ALT level (x ULN)	1.1 (0.7-2.4)	1.9 (1.2-4.3)	0.9 (0.6-1.8)	<0.001	1.7 (1.0-3.3)	1.6 (0.8-3.7)	0.086
Baseline GGTP level (IU/L)	28 (16-59)	39 (24-72)	24 (14-52)	<0.001	34 (23-64)	34 (18-68)	0.088
Baseline total bilirubin level (mg/dL)	0.7 (0.5-0.9)	0.7 (0.5-1.0)	0.6 (0.5-0.9)	<0.001	0.7 (0.5-1.0)	0.7 (0.5-0.9)	0.210
Baseline serum albumin level (g/L)	4.2 (3.9-4.5)	3.9 (3.6-4.1)	4.4 (4.1-4.6)	<0.001	3.9 (3.7-4.2)	4.0 (3.8-4.3)	0.084
†Platelet count (10^5/mm^3) (SD)	19.1 (6.3)	16.9 (5.6)	20.0 (6.4)	<0.001	17.5 (5.2)	17.2 (6.0)	0.349
Follow-up duration (yrs)	5.4 (3.1-13.2)	3.2 (2.1-4.3)	9.5 (4.4-16.1)	<0.001	3.3 (2.3-4.3)	7.6 (3.4-13.7)	<0.001
Person-years of follow-up	13,986	1561	12381	–	1064	2978	–
No. of HCC cases	156	12	144	–	6	72	–
Incidence rates per 1000 person-years	11.15	7.69	11.63	–	5.63	24.1	–
Progression of cirrhosis within 5 year	21 (1.3)	0	21 (1.8)	0.001	0	10 (3.2)	0.001
HBV DNA <400 copies/mL at 1 yearr	–	421 (89)	NA	–	288 (90)	NA	–
Emergence of drug-resistant mutants during ETV treatment	–	4 (0.8)	NA	–	2 (0.6)	NA	–

HBeAg, hepatitis B e antigen; HBV, hepatitis B virus; AST, aspartate aminotransferase; GGTP, gamma glutamyltransferase (ULN=33 IU/L); ALT, alanine amino-transferase (ULN=42 IU/L for men and 27 IU/L for women); HCC, hepatocellular carcinoma; ETV, entecavir.
*P <0.05.
**P <0.001, comparison of entecavir-treated group and control group.
†Data displayed as mean ± standard deviation.   ‡All other values are expressed as median (25th to 75th percentile) or number (percentage of total, %).

matched control group were 4.0% at year 2, 7.2% at year 3, 10.0% at year 4, and 13.7% at year 5. Log-rank test revealed a statistically significant difference between the incidence of HCC in the ETV group and the control group over time ($P < 0.001$) (Fig. 2). We then used Cox proportional regression analysis to estimate the effects of ETV treatment on HCC risk. Factors that were associated with HCC at year 5 in the propensity score matched cohort were age, gender, alcohol consumption (>200 kg), the presence of cirrhosis, HBeAg positivity, baseline viral load, ALT, γ-GTP, total bilirubin, serum albumin, and platelet counts (Table 2). For ETV treatment effect, we estimated the hazard ratio of HCC development, adjusting for multiple baseline variables (age, gender, alcohol consumption, smoking, preexisting cirrhosis, HBeAg, HBV DNA, ALT, albumin, γ-GTP, total bilirubin, and platelet count) in the propensity matched cohort. Pro-

gression of cirrhosis within 5 years was used as a time-dependent covariate in the proportional hazard regression but it did not show a statistically significant hazard to HCC development.

Subanalyses Showing HCC Suppression Effect Between ETV and LAM. PS matching of the LAM-treated patients without rescue therapy (n = 492) with ETV-treated patients resulted in a matched cohort of 182 patients (Supporting Table 3). The rate of non-rescued LAM-treated group having undetectable HBV DNA at 1 year after treatment was lower when compared with the ETV-treated group. The LAM-treated group also had a higher drug-resistant mutation rate. Comparisons of HCC incidence among the ETV-treated group, nonrescued LAM-treated group, and control showed that the HCC suppression effect was greater in ETV-treated ($P < 0.001$) than nonrescued LAM-treated ($P = 0.019$) when compared with the
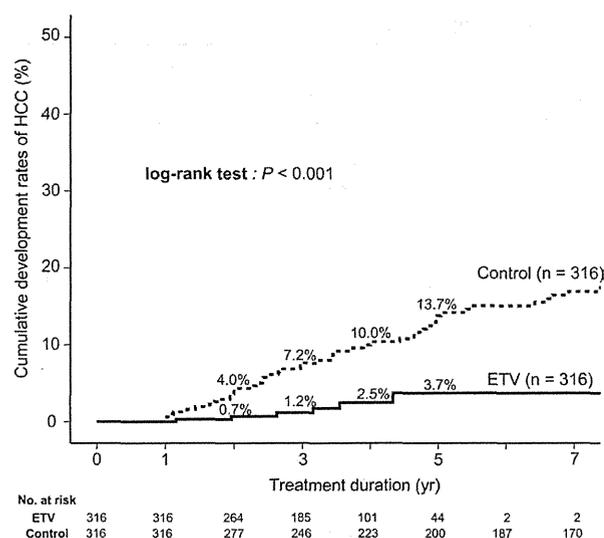
Fig. 2. Comparison of HCC cumulative incidence rates between the entecavir-treated group and the nontreated control group after propensity score matching. The log-rank test revealed a statistically significant difference between the ETV and the control group in the incidence of HCC at 5 years time (log-rank test: $P < 0.001$).

control group (Fig. 3). The difference of effect between ETV and LAM was also significant ($P = 0.043$). The treatment effect was seen in cirrhosis patients but not in noncirrhosis patients. The result showed ETV's superiority to LAM in suppressing HCC.

Effect of ETV on the Reduction of HCC Development by Preexisting Cirrhosis and Risk Scores. To further examine the ETV treatment effect, we compared the ETV and the control groups by preexisting cirrhosis and published risk scores. Viral response rates

(HBV DNA < 400 copies/mL) of 1-year post-ETV treatment was 87% in the noncirrhosis patients and 91% in the cirrhosis patients (LC). ALT normalization was 94% and 90% in the chronic hepatitis and cirrhosis patients, respectively. The treatment effect was not inferior by cirrhosis status. Among those who developed HCC, 97 out of 144 patients in the control group and 9 out of 12 patients in the ETV group had cirrhosis. Interactions between preexisting cirrhosis and ETV treatment were not observed ($P = 0.177$).

Cumulative HCC incidence rates by risk scores are compared between the two cohorts in Fig. 4A-G. Figure 4A,B shows the risk scores developed by Yang et al.[10] Figure 4C,D shows the risk scores developed by Yuen et al.[11] Figure 4E-G shows the risk scores developed by Wong et al.[12] All three risk score scales showed that ETV significantly reduced HCC incidence in patients with a higher risk (risk score ≥ 12, $P = 0.006$; risk score ≥ 82, $P = 0.002$; medium risk, $P = 0.062$; high risk, $P < 0.001$). Interactions between risk scores and ETV treatment were not observed (Yang et al.: $P = 0.713$, Yuen et al.: $P = 0.267$, Wong et al.: $P = 0.265$).

Discussion

Our study suggests that long-term ETV therapy would significantly suppress the development of HCC in HBV-infected patients when compared with HBV-infected patients in the control group. The treatment effect was more prominent among patients at high risk of HCC than those at low risk.

Table 2. Factors Associated with HCC Development as Determined by Cox Proportional Hazard Regression Analysis at 5-Year (Propensity Score Matched Cohort)

Variable	Univariate HR (95% CI)	P	Multivariate Adjusted HR (95% CI)	P
Age (per year)	1.05 (1.02-1.07)	<0.001	1.06 (1.03-1.09)	<0.001
Gender (M)	2.81 (1.25-6.32)	0.012		
Alcohol consumption (>200kg)	2.71 (1.49-4.92)	0.001	2.21 (1.18-4.16)	0.013
Cigarette smoking	1.53 (0.84-2.80)	0.164		
Preexisting cirrhosis	12.0 (5.57-25.9)	<0.001	4.28 (1.88-9.73)	0.001
HBV genotype (C)	2.73 (0.98-7.65)	0.056		
HBeAg (positive)	2.64 (1.41-4.94)	0.002	2.26 (1.18-4.34)	0.014
HBV DNA ($\geq$5.0 log copies/mL)	4.66 (1.44-15.1)	0.010		
ALT ($\geq$45 IU/L)	2.29 (1.10-4.77)	0.027		
GGTP ($\geq$50 IU/L)	3.79 (2.02-7.09)	<0.001		
Total bilirubin ($\geq$1.5 mg/dL)	5.51 (2.87-10.6)	<0.001		
Serum albumin (<3.8 g/L)	4.44 (2.42-8.14)	<0.001		
Platelet count (<1.5×10^5/mm^3)	14.8 (5.84-37.7)	<0.001	5.64 (2.13-15.0)	0.001
*Progression of cirrhosis within 5 years	1.80 (0.25-13.2)	0.562		
ETV treatment	0.23 (0.09-0.55)	0.001	0.37 (0.15-0.91)	0.030

Asterisks (*) indicate time-dependent covariates.

†Adjusted for age, gender, alcohol, cigarette, cirrhosis, genotype, HBeAg, HBV DNA, ALT, albumin, GGTP, total bilirubin, and platelet counts

Abbreviations: ETV, entecavir; HR, hazard ratio; CI, confidence interval; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; GGTP, gamma glutamyltransferase.
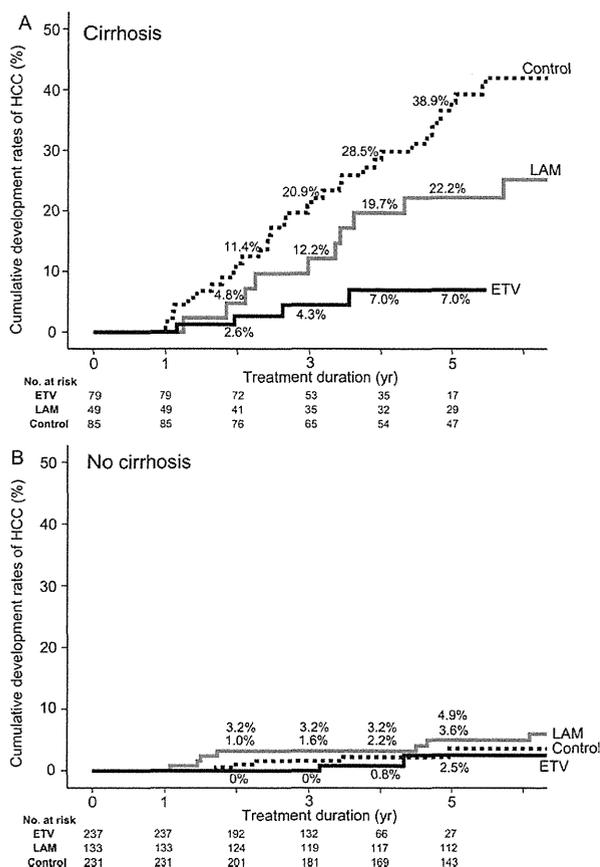
Fig. 3. Comparison of HCC cumulative incidence rates between the entecavir (ETV)-treated group, lamivudine (LAM)-treated, and the non-treated control group after PS matching stratified by cirrhosis. The log-rank test revealed a statistically significant difference in the incidence of HCC at 5 years time in cirrhosis patients: ETV versus control group ($P < 0.001$); LAM versus control ($P = 0.019$); ETV versus LAM ($P = 0.043$). The differences were not seen in the noncirrhosis patients: ETV versus control ($P = 0.440$); LAM versus control ($P = 0.879$); ETV versus LAM ($P = 0.126$).

HBV has been previously shown to influence HCC development. Ikeda et al.[20] reported that the cumulative HCC incidence rates among Japanese HBV patients were 2.1% at 5 years, 4.9% at 10 years, and 18.8% at 15 years among NA-naïve patients. Other studies, both from Japan and other countries, have reported a 5-year cumulative HCC incidence rate of 3.3% among chronic HBV, and 21.2% to 59% among cirrhosis patients.[21,22] The incidence of HCC varies significantly by country and ethnic group,[4] which seems to be attributable to diverse exposure to HCC risk factors.

Carcinogenicity related to HBV infection is somewhat complex and multifactorial when compared with carcinogenicity related to HCV infection. Known HCC risk factors among HBV-infected patients include older age, male gender, cirrhotic status, diabetes mellitus, family history, alcohol consumption, AST,

HBsAg, HBeAg, and genotype C.[20,23,25] Chen et al.[5] found a dose-response relationship between pretreatment serum HBV DNA levels and the development of HCC. Baseline ALT is another risk factor for HCC, as elevated ALT levels indicate an active immune response against HBV, resulting in repetitive hepatocyte injury.[5] Our study corroborates these findings on these factors influence on HCC development.

The potential ability of ETV to reduce the risk of HCC is an additional example of a long-term NA treatment effect. Some studies have shown that ETV has low incidence of HCC but these studies did not have a control arm.[9] A meta-analysis and a systematic review showed that NAs can reduce liver complications, including HCC.[26,27] Other studies have begun to show that control of sustained viral loads through drugs such as NAs is important in preventing long-term complications. Chen et al.[28] showed that greater decreases in serum HBV DNA levels ($<10^4$ copies/mL) during follow-up were associated with a lower risk of HCC.

Our comparison among the PS-matched ETV-treated group, nonrescued LAM-treated patients, and the control showed that ETV is superior to LAM in HCC suppression. Kurokawa et al.[29] showed that treatment with lamivudine for an average of 5 years reduced the incidence of HCC in HBV-infected cirrhosis patients, who showed sustained viral response at a median HBV DNA of <4.0 log copies/mL. Unfortunately, only 48% of the patients in this study achieved sustained viral response, while 51% developed lamivudine-resistant tyrosine-methionine-aspartate-aspartate mutation (YMDD mutation) during follow-up.[29] Patients with drug resistance were reported to have a 2.6 times greater chance of developing long-term complications.[26] A systematic review of 21 studies showed that HCC occurred more (2.3% versus 7.5%, $P < 0.001$) in nonresponding patients or in patients with viral breakthrough compared with those who experienced remission.[28] On-treatment drug resistance could subject patients to a variable viral status. Suppression of HCC by NAs requires NAs that do not lead to drug resistance. Compared with other NAs, ETV shows minimal drug resistance. Our results showed that ~90% of the ETV-treated patients had sustained viral suppression at year 1, and that drug resistance was minimal (0.8%) during the median follow-up period of 3.2 years.

We found that the effect of ETV treatment in reducing the risk of HCC was more prominent among high-risk patients. This phenomenon was observed by examining the combination of parameters associated with the recently developed risk scores (Fig. 4). The published risk scores were developed mainly to create
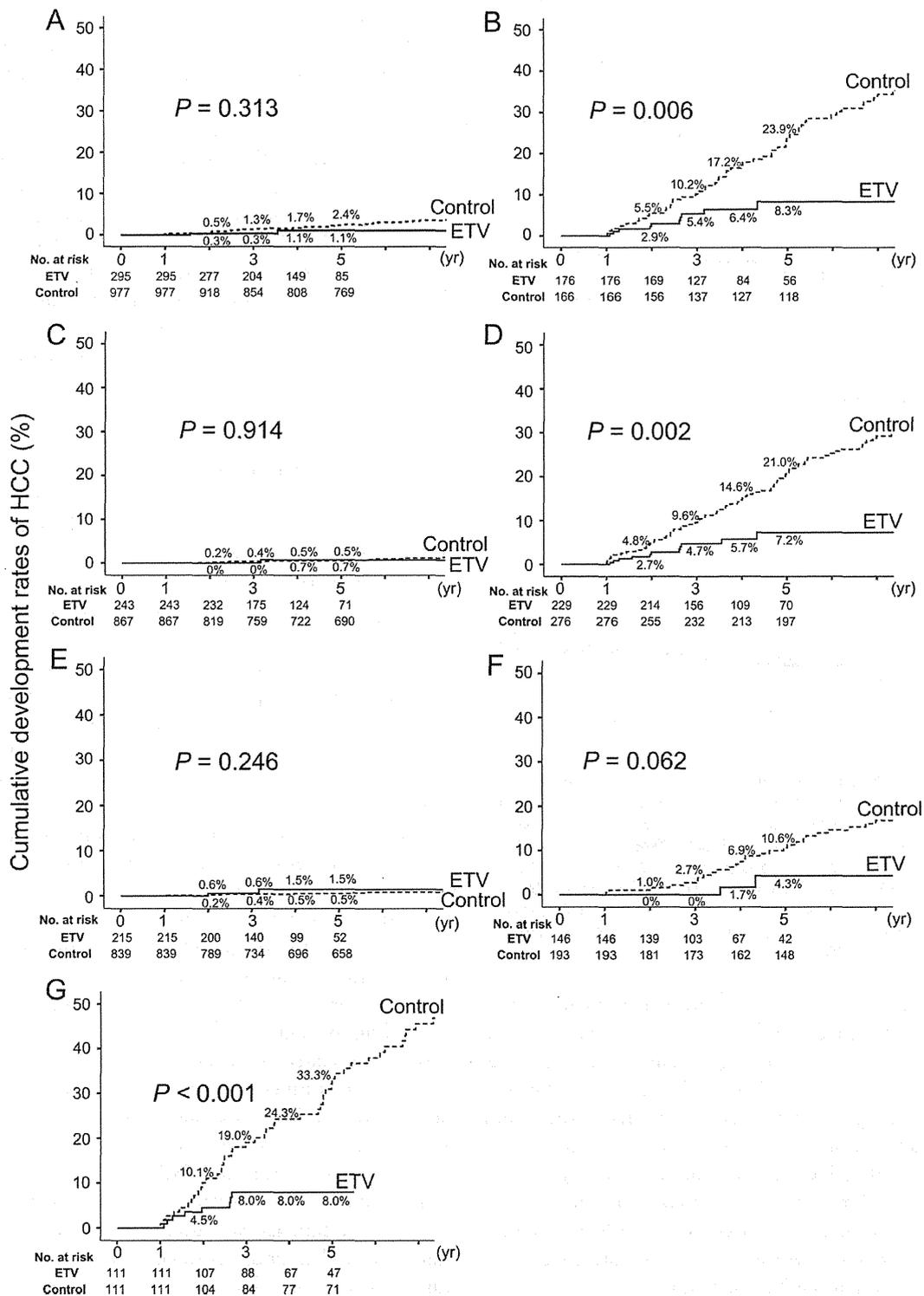
Fig. 4. Cumulative incidence of HCC by risk score scales: comparison between entecavir-treated and nontreated control patients: Risk score cutoff points were based on those presented in articles by the following: A,B (Yang et al.[10]): low-risk score cutoff point < 12; high-risk score cutoff point ≥ 12. C,D (Yuen et al.[11]): low-risk score cutoff point < 82; high-risk score cutoff point ≥ 82. E-G (Wong et al.[12]): low-risk score cutoff point < 4; medium-risk cutoff point 4-19; high-risk score cutoff point ≥ 20. A statistically significant difference in HCC incidence was seen between the ETV group and the control group in the higher-risk groups when observed the incidence of HCC over time (log-rank test P = 0.006 for risk score ≥ 12; P = 0.002 for risk score ≥ 82; P = 0.062 for patients with medium risk; P < 0.001 for patients at high risk for HCC).

easy-to-use nomograms based on clinical characteristics to predict the risk of HCC in patients with HBV. These scales have been validated, and can accurately estimate the risk of HCC up to 10 years. The cutoff scores used in these studies were based on their sensitivity to detect HCC derived and validated with non-treated HBV cohorts. The importance of our study using these risk scales in our cohorts was to see the change in risk with the initiation of therapy. We found that the ETV treatment effect to reduce the risk of HCC was more prominent among cirrhosis and high-risk patients despite the lack of interactions between ETV treatment and preexisting cirrhosis or risk factors. The lower treatment effect among lower-risk patients was somewhat not surprising. HCC development among low-risk patients is generally rare, and therefore, the treatment effect may not have occurred in large enough numbers during the treatment period allotted in our study to be able to detect a difference. In addition, HCC development differs greatly by cirrhotic status and risk factors in the control group. The treatment effect of ETV to reduce HCC is probably more likely reflected among cirrhosis or high-risk patients. A study with a longer observation period and higher patient numbers might be necessary to examine this ETV treatment effect among low-risk patients. The development of a scoring system to predict treatment effect of HBV patients with different risk levels will be useful in determining the most appropriate timing of treatment initiation in clinical settings.

Study Limitations. There were several limitations to our study. First, because our patients were recruited from one hospital, they might not have been representative of the general Japanese HBV population. Second, our control group included historically observed patients who entered the cohort long before the ETV group, resulting in treatment differences during the time gap. However, we used PS matching and a similar follow-up period between the two cohorts to minimize this bias. Third, our study was an observational study with patients having large demographic differences. Although we used a PS to match ETV-treated and control groups, our sample size did not take into account other unobserved confounding factors such as HCC family history, stage of cirrhosis, and comorbidities when determining associating factors for carcinogenesis in HBV. Finally, the observation period of the ETV group was relatively short, and patients in the ETV-treated cohort at 5 years consisted of only less than ~25% of the initial recruited patients. Because of this limitation, we censored patients who were followed for more than 5 years. The observed treatment effect would require confirmation over a longer period and a more complete follow-up.

Conducting a long-term study to examine the effect of antiviral therapy with HCC as the endpoint would be time-consuming and challenging. Such a study would require a large sample size and would, therefore, be costly. In addition, the increases in choices of therapy over time would make it difficult to conduct a long-term study using a single therapy. Owing to ethical issues, it would be difficult to recruit or follow a naïve, untreated cohort over an extended period of time. Because of these challenges, most studies have examined the relationship between antiviral treatment and the risks of HCC involved older drugs, lacked a control group, or were of relatively short duration. Consequently, the association between antiviral treatment and carcinogenesis is inferential and requires additional confirmatory studies.

In conclusion, in our study we observed the effect of HCC risk among HBV-infected patients treated by ETV by comparing them with a group of NA-naïve patients. We followed these Japanese patients for a relatively long period of time and compared them with a large pool of untreated control patients. In this long-term study among Japanese patients, ETV significantly reduced the incidence of HCC among chronic HBV-infected patients, and was more prominent among patients at higher risk for HCC.

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Original Article

Fibrosis score consisting of four serum markers successfully predicts pathological fibrotic stages of chronic hepatitis B

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Aim: In order to evaluate and judge a fibrotic stage of patients with chronic hepatitis B, multivariate regression analysis was performed using multiple fibrosis markers.

Method: A total of 227 patients from seven hepatology units and institutes were diagnosed by needle biopsy as having chronic liver disease caused by hepatitis B virus. Twenty-three variables and their natural logarithmic transformation were employed in the multivariate analysis. Multiple regression function was generated from data of 158 patients in one hospital, and validation was performed using the other data of 69 patients from six other hospitals.

Results: After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times \ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3$/mm^3) $+ 1.24 \times \ln$ (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) $+ 1.19 \times \ln$ (α-2-macroglobulin)

(mg/dL) $- 9.15$. Median values of fibrosis scores of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively. Multiple regression coefficient and coefficient of determination were 0.646 and 0.418, respectively. Validation with patient data from other institutions demonstrated good reproducibility of fibrosis score for hepatitis B (FSB), showing 1.33 in F1 ($n = 27$), 2.20 in F2 ($n = 20$), 3.11 in F3 ($n = 20$) and 5.30 in F4 ($n = 2$), respectively.

Conclusion: A concise multiple regression function using four laboratory parameters successfully predicted pathological fibrosis stage of patients with hepatitis B virus infection.

Key words: chronic hepatitis, hepatitis B virus, liver cirrhosis, liver fibrosis, multiple regression analysis, stage

INTRODUCTION

WHEN HEPATITIS B virus (HBV)-related chronic liver disease is found by biochemical and virological examination, liver biopsy can establish the definitive diagnosis of chronic hepatitis and its fibrotic staging. Although these pathological procedures are reliable and informative both in diagnosis and treatment, they sometimes require medical invasion and financial costs, including the risk of bleeding from needle puncture, some pain experienced during the procedure and hospital stays of a few days. The pathological examination is, therefore, rarely performed repeatedly in a short period of time, unless disease activity is severe or progression of liver disease is highly suspected. Recently, many authors described the usefulness of ultrasonographic elastography and multiple resonance imaging technology in the estimation of staging of chronic hepatitis and cirrhosis.[1–5] These ways of estimation using the imaging apparatuses seem truly useful for current patients, but they cannot evaluate and compare with past fibrotic states of patients retrospectively. Moreover,

the same apparatus for elastometry will not be available for repeated measurement for a follow-up examination, for example, several years later.

In spite of the accuracy of biopsy and convenience of elastography in chronic liver disease, clinical diagnosis based on biochemistry and hematology is still indispensable for the daily practice of many patients with HBV-related liver disease. Recently, several studies were published about estimation of hepatitis stages, using one or more serum biomarkers. Discriminant functions or multivariate analyses demonstrated that approximately 60–90% of patients with chronic hepatitis B were correctly classified as having mild hepatitis and severe hepatitis with advanced fibrosis.[2,6-13] Up to the present time, however, the usefulness of the discriminant functions are less valuable for a few reasons. First, these functions were made for the purpose of discrimination of severe hepatic fibrosis from mild fibrosis, and four histological classifications (F1–F4) were neglected in almost of the studies. Second, some studies analyzed both hepatitis B and hepatitis C virus infection, although the significance and actual values of each liver function test in the evaluation of the severity of liver disease were not similar among each viral hepatitis and alcoholic liver disease. Third, biochemical markers for liver fibrosis (e.g. hyaluronic acid, type IV collagen, procollagen III peptide)[14-16] were not always included in those previous studies.

We tried to generate a function estimating fibrotic stages of HBV-related chronic hepatitis, which were objectively diagnosed by liver biopsy. The purpose of this study is, therefore, to make a reliable multiple regression function and to obtain practical coefficients for significant variables also using fibrosis markers.

METHODS

Patients

A TOTAL OF 273 Japanese patients with chronic hepatitis B were recruited for the study from seven hospitals in Japan: Toranomon Hospital, Hiroshima University Hospital (K. Chayama, M.D.), Ehime University Hospital (M. Onji, M.D.), Musashino Red Cross Hospital (N. Izumi, MD), Shishu University Hospital (E. Tanaka, M.D.), Showa University Hospital (M. Imawari, M.D.) and Osaka University Hospital (T. Takehara, M.D.). Inclusion criteria for this study were: (i) positive hepatitis B surface antigen for more than 6 months; (ii) persistent or intermittent elevation in aspartate aminotransferase (AST)/alanine aminotransferase (ALT) levels; and (iii) liver biopsy showing chronic hepatitis

(F1–F4). We excluded those patients with overt alcoholic liver disease or fatty liver, association of other types of liver disease (e.g. hepatitis C, primary biliary cirrhosis, autoimmune hepatitis), or those associated with hepatocellular carcinoma or other malignancy. Among the patients, 244 patients fulfilled the conditions for the study: complete demographic data, basic laboratory data of hematology and biochemistry, required liver biopsy specimens, and sufficient amount of frozen sera. Also, we excluded additional 17 patients with eventual histological diagnosis as F0 stage.

Finally, a total of 227 patients who were diagnosed as having chronic hepatitis or cirrhosis (F1–F4) were analyzed for the following hematological, biochemical and histopathological examination. There were 172 males and 55 females aged 16–70 years (median, 39 years).

All the patients presented written informed consent in individual hospitals and medical centers, and the study was approved in each ethical committee.

Hematological and biochemical examination

Hematological and standard biochemical evaluation had been performed in each medical institution: white blood cells, red blood cells, hemoglobin, platelets, total bilirubin, AST, ALT, AST/ALT ratio (AAR), γ-glutamyl transpeptidase (γ-GTP), total protein, albumin and γ-globulin.

Special biochemical examinations including "fibrosis markers" were carried out using stored frozen sera at $-20°C$ or lower: α-2-macroglobulin, haptoglobin concentration, haptoglobin typing, apolipoprotein A1, hyaluronic acid, tissue inhibitor of matrix metalloproteinase (TIMP)-1, TIMP-2, procollagen III peptide and type IV collagen 7S.

Histological diagnosis of chronic hepatitis and cirrhosis

All the 227 cases fulfilled required standards of histological evaluation: sufficient length of specimen, hematoxylin–eosin staining, and at least one specimen with fiber staining. Four independent pathologists (Y. T., J. F., F. K. and T. F.), who were not informed of patients' background and laboratory features except for age and sex, evaluated the 227 specimens regarding the stages of fibrosis and activity. Pathological classification of chronic hepatitis staging was based on Desmet *et al.*[17]

Before judgment of histological staging of individual specimens, the pathologists discussed the objective and reproducible judgment of pathological diagnosis of

hepatitis. They made a panel about obvious criteria using typical microscopic pictures for each stage, and it was always referred to during the procedure of pathological judgment. When inconsistent results were found in the diagnosis of hepatitis stage among the pathologists, the final judgment accepted majority rule among them.

Statistical analysis

Non-parametric procedures were employed for the analysis of background characteristics and laboratory data among patients in each stage, including Mann–Whitney U-test, Kruskal–Wallis test and χ^2-test.

The normality of the distribution of the data was evaluated by a Kolmogorov–Smirnov one-sample test. Because certain variables partly did not conform to a normal distribution, natural logarithmic transformation of bilirubin, AST, ALT, γ-GTP, α-2-macroglobulin, hyaluronic acid, type IV collagen 7S and TIMP-2 were also analyzed in the following calculation. The natural logarithmic transformation of the results yielded a normal distribution or symmetrical distribution for all the analyzed factors. After the procedures, the following multiple regression analysis became rationally robust against deviations from normal distribution. In order to avoid introducing into the model any variables that were mutually correlated, we checked the interaction between all pairs of the variables by calculating variance inflation factors. Of the highly correlated variables, less significant factors were removed from the viewpoint of multicollinearity.

Multivariate regression analysis was performed using 158 patient data from Toranomon Hospital (training dataset) to generate a training data of predicting function. We used a stepwise method for selection of informative subsets of explanatory variables in the model. Multiple regression coefficient and coefficient of determination were also taken into account in the selection of variables. Next, we validated the obtained predictive function using the remaining 69 patient data from the other six liver institutions (validation dataset).

A P-value of less than 0.05 with two-tailed test was considered to be significant. Data analysis was performed using the computer program SPSS ver. 19.[18]

For evaluation of the efficiency and usefulness of obtained function for fibrosis estimation, we compared various fibrosis scores for hepatitis B and C, including AAR,[19] AST-to-platelet ratio index (APRI),[20] FIB-4,[21] FibroTest[22] and discrimination function of cirrhosis from hepatitis in Japanese patients.[23]

RESULTS

Pathological diagnosis

FOUR PATHOLOGISTS INDEPENDENTLY judged the fibrotic stages and inflammatory activity for 227 specimens of chronic hepatitis/cirrhosis caused by HBV. One hundred patients (44.1%) had a fibrosis stage of F1, 62 (27.3%) F2, 51 (22.5%) F3 and 14 (6.2%) F4. In the subgroup of the 158 patients in the training group, judgment as F1 was made in 73 cases, F2 in 42, F3 in 31 and F4 in 12. Of the 69 patients in the validation group, judgment as F1 was made in 27, F2 in 20, F3 in 20 and F4 in two.

According to hepatitis activity classification, A0 was found in five (2.2%), A1 in 100 (44.1%), A2 in 107 (47.1%) and A3 in 15 (6.6%).

Laboratory data of each hepatitis stage in the training group

There were 124 men and 34 women with a median age of 39 years ranged 16–70 years. Laboratory data of 158 patients in the training group are shown in Table 1. Although several individual items were well correlated with the severity of hepatic fibrosis, significant overlap values were noted among F1–F4 stages: platelet count, γ-globulin, α-2-macroglobulin, haptoglobin, hyaluronic acid, TIMP-2 and type IV collagen 7S.

Significant variables serving staging of hepatitis

Univariate analyses using trend analysis with the Cochran–Armitage method showed that the fibrotic stage of chronic hepatitis B (FSB) was significantly correlated with platelet count (Spearman: $r = -0.45$, $P < 0.001$), γ-GTP ($r = 0.19$, $P = 0.017$), γ-globulin ($r = 0.29$, $P < 0.001$), α-2-macroglobulin ($r = 0.32$, $P < 0.001$), hyaluronic acid ($r = 0.36$, $P < 0.001$), TIMP-2 ($r = 0.16$, $P = 0.043$), procollagen III peptide ($r = 0.30$, $P < 0.001$) and type IV collagen 7S ($r = 0.55$, $P < 0.001$).

Regression function generated from training patient group

After stepwise variable selection, multivariate regression analysis finally obtained the following function: $z = 1.40 \times ln$ (type IV collagen 7S) (ng/mL) $- 0.017 \times$ (platelet count) ($\times 1000^3/mm^3$) $+ 1.24 \times ln$ (TIMP-2) (ng/mL) $+ 1.19 \times ln$ (α-2-macroglobulin) (mg/dL) $- 9.15$. Median values of the fibrosis score of F1 ($n = 73$), F2 ($n = 42$), F3 ($n = 31$) and F4 stages ($n = 12$) were calculated as 0.95, 2.07, 2.98 and 3.63, respectively

Table 1 Demography and laboratory data of 158 patients in training group

	F1 ($n = 73$)	F2 ($n = 42$)	F3 ($n = 31$)	F4 ($n = 12$)
Demographics				
Men : women	58:15	33:9	23:8	10:2
Age (median, range)	36 (16–70)	39.5 (18–66)	39 (25–64)	43 (32–59)
Laboratory data (median, range)				
WBC ($\times 1000/mm^3$)	5.4 (2.5–10.6)	5.1 (2.4–8.7)	4.9 (3.0–8.7)	4.1 (3.7–6.6)
Hemoglobin (g/dL)	15.3 (10.3–18.8)	15.4 (12.5–17.9)	15.2 (11.5–17.2)	14.45 (12.1–18.2)
Platelet ($\times 1000/mm^3$)	204 (124–341)	173 (82–308)	155 (96–220)	130 (86–230)
Albumin (g/dL)	4.1 (3.2–4.9)	4.0 (3.2–5.1)	4.0 (3.3–4.9)	3.95 (3.4–4.6)
Bilirubin (mg/dL)	0.8 (0.2–1.7)	0.8 (0.3–2.3)	0.9 (0.4–5.4)	0.85 (0.6–2.3)
AST (IU/L)	48 (16–450)	55 (17–588)	54 (17–1446)	76.5 (27–396)
ALT (IU/L)	102 (10–839)	90 (12–886)	85 (19–2148)	89 (18–809)
γ-GTP (IU/L)	37 (7–247)	55 (8–687)	44 (14–564)	69 (33–262)
γ-Globulin (g/dL)	1.29 (0.78–2.11)	1.495 (0.62–3.20)	1.43 (0.90–2.30)	1.735 (0.92–2.47)
γ-Globulin (%)	17.3 (10.8–26.1)	19.3 (8.5–35.6)	19.9 (12.9–28.6)	22.55 (13.9–30.2)
α-2-Macroglobulin (mg/dL)	226 (116–446)	276 (148–495)	261 (202–565)	286.5 (166–425)
Haptoglobin (mg/dL)	77 (<5–318)	59 (<5–238)	61 (<5–151)	48.5 (<5–145)
Apolipoprotein A-I (mg/dL)	134 (89–212)	143 (78–250)	133 (87–189)	125 (73–169)
Hyaluronic acid (μg/L)	16 (<5–130)	32.5 (<5–204)	38 (<5–418)	49 (24–335)
TIMP-1 (ng/mL)	168 (93–271)	172 (116–314)	157 (119–365)	192 (145–365)
TIMP-2 (ng/mL)	80 (41–135)	80.5 (35–121)	92 (38–251)	85.5 (70–123)
Procollagen III peptide (U/mL)	0.75 (0.53–1.90)	0.835 (0.45–1.20)	0.89 (0.58–2.50)	1.05 (0.71–2.20)
Type IV collagen 7S (ng/ml)	4.0 (2.7–7.7)	4.6 (2.6–9.6)	5.6 (2.3–15.0)	7.2 (4.2–14.0)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cells.

(Fig. 1). The multiple regression coefficient and coefficient of determination were 0.646 ($P < 0.001$) and 0.418 ($P < 0.001$), respectively.

Because the generated regression function was obtained by multivariate analysis with stepwise variable selection, several variables were removed from the function due to multicollinearity among them. Mutual correlation among the fibrosis predictors are shown in Table 2.

A 28-year-old man of F1 fibrotic stage (Fig. 2a) had a serum type IV collagen concentration of 4.4 ng/mL, platelet 221×10^3 count/mm^3, TIMP-2 75 ng/mL and α-2-macroglobulin 226 mg/dL. The regression function provided a fibrosis score of 0.99. Another man aged 46 years had F3 fibrosis on histological examination (Fig. 2b). His type IV collagen was 5.3 ng/mL, platelet 137×10^3 count/mm^3, TIMP-2 92 ng/mL and α-2-macroglobulin 255, and the regression function calculated his fibrosis score as 3.10.

Validation of discriminant function

Validation data of 69 patients (Table 3) were collected from the other six institutions in Japan. When applying
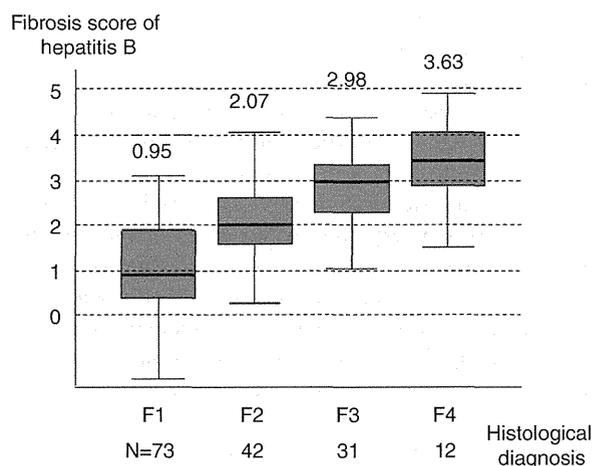
Figure 1 Box and whisker plots of fibrotic score of each histological fibrosis group in the training dataset. The fibrosis score of hepatitis B was generated by the function, z = 1.40 × *ln* (type IV collagen 7S) (ng/mL) – 0.017 × (platelet count) ($\times 1000^3/mm^3$) + 1.24 × *ln* (tissue inhibitor of matrix metalloproteinase-2) (ng/mL) + 1.19 × *ln* (α-2-macroglobulin) (mg/dL) – 9.15.

Table 2 Correlation coefficients (Spearman's ρ) among fibrosis predictors used in multivariate analysis

	Platelet	gamma-globulin	ln (α-2-macroglobulin)	ln (hyaluronate)	ln (P-III-P)	ln (IV collagen)	ln (TIMP-2)
Platelet (×10³/mm³)	1.000	−0.214 ($P = 0.008$)	−0.260 ($P = 0.001$)	−0.384 ($P < 0.001$)	−0.045 ($P = 0.58$)	−0.297 ($P < 0.001$)	0.094 ($P = 0.24$)
γ-Globulin (g/dL)		1.000	0.276 ($P = 0.001$)	0.349 ($P < 0.001$)	0.342 ($P < 0.001$)	0.414 ($P < 0.001$)	0.268 ($P = 0.001$)
ln (α-2-macroglobulin) (mg/dL)			1.000	0.281 ($P < 0.001$)	0.141 ($P = 0.078$)	0.171 ($P = 0.032$)	−0.079 ($P = 0.32$)
ln (hyaluronic acid) (mg/L)				1.000	0.373 ($P < 0.001$)	0.493 ($P < 0.001$)	0.089 ($P = 0.27$)
ln (procollagen III peptide) (U/mL)					1.000	0.600 ($P < 0.001$)	0.145 ($P = 0.071$)
ln (type IV collagen) (mg/L)						1.000	0.358 ($P < 0.001$)
ln (TIMP-2) (mg/L)							1.000

TIMP, tissue inhibitor of matrix metalloproteinase.

the regression function for the validation set, the fibrosis score demonstrated good reproducibility, showing 1.33 in patients with chronic hepatitis of F1 ($n = 27$), 2.20 of F2 ($n = 20$), 3.11 of F3 ($n = 20$) and 5.30 of F4 ($n = 2$), respectively (Fig. 3). Although F4 fibrosis stage consisted of only two patients and the score 5.30 was regarded as of rather higher value, the scores of other stages of fibrosis were concordant with histological fibrosis.

Comparisons of efficacy with various fibrosis scores (Fig. 4)

In order to evaluate the efficacy and usefulness of the obtained FSB, we compared it with previously reported fibrosis scores using training data. AAR, APRI and FibroTest showed only slight correlation with actual histological stage. FIB-4 demonstrated an increasing trend of the score associated with histological fibrosis, but significant overlapping scores were found in F1–F4. Spearman's correlation coefficients of AAR, APRI, FIB-4 and FibroTest were 0.199 ($P = 0.012$), 0.265 ($P = 0.001$), 0.412 ($P < 0.001$) and 0.330 ($P < 0.001$), respectively. Our FSB showed a Spearman's correlation coefficient of 0.625 ($P < 0.001$), and was a much higher value than the others. The dichotomous discrimination function for cirrhosis and hepatitis C in Japanese patients[23] showed good differentiation also in patients with hepatitis B virus.

DISCUSSION

RECOGNITION OF SEVERITY of chronic hepatitis is essential in managing patients with chronic HBV infection: estimation of length of infection, existence of any previous hepatitis activity, presumption of current fibrotic stage, and prediction of future fibrosis progression and hepatocarcinogenesis. Differential diagnosis of cirrhosis from chronic hepatitis is especially important in the evaluation of chronic HBV infection. Identification of liver cirrhosis often leads to an important change in management of the patient: need for fiberscopic examination for esophageal varices, ultrasonographic exploration for the association of liver cancer, and prediction of hepatic decompensation. Guidelines published by the American Association of Study of Liver Disease[24] recommend liver biopsy for HBV carriers with aminotransferase elevation or for any candidates of antiviral therapy, because hepatic fibrosis sometimes shows unexpectedly far advancement to cirrhosis, and because it is very difficult to evaluate and translate the liver function tests or ultrasonographic findings compared to chronic hepatitis type C.

Table 3 Demography and laboratory data of 69 patients in training group

	F1 ($n = 27$)	F2 ($n = 20$)	F3 ($n = 20$)	F4 ($n = 2$)
Demographics				
Men : women	18:9	15:5	13:7	2:0
Age (median, range)	36 (13–64)	45 (14–64)	36.5 (24–59)	32 (25–39)
Laboratory data (median, range)				
WBC ($\times 1000/mm^3$)	5.0 (2.8–8.7)	5.8 (2.8–11.6)	5.3 (3.2–8.1)	3.85 (2.7–5.0)
Hemoglobin (g/dL)	14.8 (12.4–17.4)	15.0 (12.4–16.9)	14.4 (11.1–16.4)	14.4 (12.5–16.3)
Platelet ($\times 1000/mm^3$)	204 (86–322)	180 (90–275)	147 (90–276)	130 (67–183)
Albumin (g/dL)	4.4 (2.8–5.2)	4.2 (3.5–5.1)	4.3 (3.4–4.9)	4.45 (4.0–4.9)
Bilirubin (mg/dL)	0.9 (0.4–6.4)	0.8 (0.2–1.6)	0.75 (0.4–1.7)	1.15 (1.1–1.2)
AST (IU/L)	52 (17–575)	50.5 (21–272)	65 (22–284)	248.5 (51–446)
ALT (IU/L)	84 (16–1101)	101.5 (19–554)	86.5 (16–1113)	453.5 (74–833)
γ-GTP (IU/L)	42 (14–332)	54 (16–205)	52.5 (13–191)	193 (57–329)
γ-Globulin (g/dL)	1.30 (1.04–1.59)	1.35 (1.18–2.53)	1.62 (1.16–1.97)	1.545 (1.51–1.58)
γ-Globulin (%)	17.9 (14.3–22.1)	19.6 (15.5–30.8)	22.0 (16.5–24.6)	20.15 (19.3–21.0)
α-2-Macroglobulin (mg/dL)	287 (160–687)	270 (89–452)	272.5 (211–463)	389 (313–465)
Haptoglobin (mg/dL)	58 (<5–229)	74 (<5–154)	56.5 (<5–198)	<5 (<5–<5)
Apolipoprotein A-I (mg/dL)	146 (95–216)	137 (87–162)	120 (88–170)	100.5 (74–127)
Hyaluronic acid (μg/L)	27 (<5–113)	36 (10–1050)	59 (14–439)	331 (225–437)
TIMP-1 (ng/mL)	168.5 (83–302)	176 (127–408)	182 (104–303)	390.5 (283–498)
TIMP-2 (ng/mL)	76 (25–143)	86.5 (28–154)	77.5 (32–141)	100.5 (91–110)
Procollagen III peptide (U/mL)	0.71 (0.27–2.20)	0.88 (0.63–2.80)	0.995 (0.60–2.10)	1.75 (1.50–2.00)
Type IV collagen 7S (ng/ml)	3.6 (2.7–17.0)	5.25 (3.3–13.0)	5.7 (3.0–16.0)	15.5 (15.0–16.0)

ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GTP, γ-glutamyl transpeptidase; TIMP, tissue inhibitor of matrix metalloproteinase; WBC, white blood cells.

Recently, non-invasive estimation of severity of liver fibrosis has been reported in patients with HBV-related chronic hepatitis.[2,6-13] However, these studies were principally aimed at differentiation of advanced fibrotic stages of F3 or F4 from mild fibrotic stages of F1 or F2. Those discrimination functions were insufficient to recognize the stepwise progression of viral hepatitis from F1–F4. This dichotomy (mild or severe) of chronic hepatitis B seemed less valuable in the study of disease progression, disease control abilities of antiviral drugs and estimation of histological improvement after anti-inflammatory drugs. A histology-oriented, practical and reliable formula is therefore required for the diagnosis and investigation of chronic hepatitis B.

This study aimed to establish non-invasive evaluation and calculation of liver fibrosis for patients with chronic hepatitis B virus infection. Although it was retrospectively performed as a multicenter study of eight institutions, judgment of histological diagnosis was independently performed by four pathologists in another hospital, who were informed only of the patient's age, sex and positive HBV infection. Objective judgment of the histological staging and grading in sufficient biopsy specimens could be obtained.
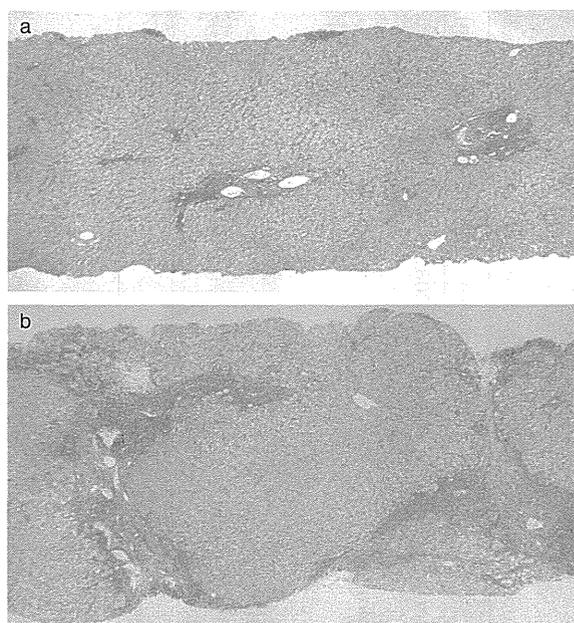
Figure 2 Case presentations of the training set. (a) A 28-year-old man with F1 fibrosis. Final regression function provided his fibrosis score as 0.99. (b) A 45-year-old man with F3 fibrosis. His regression coefficient was calculated as 3.10. Silver stain, ×40.